REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
2009	Open Literature – Book Chapter	

4. TITLE AND SUBTITLE
Botulinum toxin

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Anderson, J, Williams, PT, Katos, AM, Krasna, M, Burrows, W, Hilmas, CJ

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
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8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-P09-001

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDZ-I
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
Published in Handbook of Toxicology of Chemical Warfare Agents (ed. R.C. Gupta), 407-432, Academic Press: London, 2009.

14. ABSTRACT
See reprint.

15. SUBJECT TERMS
Botulinum intoxication, medical countermeasures, treatment, toxicology

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Corey Hilmas
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED	26	**19b. TELEPHONE NUMBER** *(include area code)* 410-436-2563

CHAPTER 30

Botulinum Toxin

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Opinions, interpretations, conclusions, and recommendations are those of the author(s) and are not necessarily endorsed by the US Army.

I. INTRODUCTION

Botulism is a disease caused by anaerobic, spore-forming bacteria found in soil. Disease results from the actions of chemical toxins produced by these bacteria. The most common forms of human botulism include foodborne, infant, and wound. The main etiology of botulism in humans is foodborne; this form is caused by eating foods contaminated with botulinum spores, which germinate and multiply into bacteria to produce neurotoxin in the food. Commonly contaminated foods include improperly preserved home-processed foods such as honey, corn, green beans, and beets. Less likely sources are fish products and other commercially processed foods. Infant botulism is often associated with eating honey contaminated with spores, but new evidence suggests that soil and dust brought into the house from the outside may be a significant source of botulinum spores. Wound botulism occurs when spores contaminate a wound, germinate, and produce toxins absorbed into the bloodstream. Regardless of the form of botulism, disease results from the intoxicating effects of potent neurotoxins.

Botulinum neurotoxins (BoNTs) comprise a family of seven distinct neurotoxic proteins produced by immunologically discrete strains of the anaerobic bacteria. These spore forming, Gram-positive bacteria secrete deadly toxins with an estimated human LD_{50} of 1–3 ng/kg (Simpson, 2004; Sobel *et al.*, 2004). In fact, BoNTs are the most potent substances known to humankind. Due to their extremely high potency, ease of production and previous history of weaponization, the BoNTs have been designated as category A threat agents by the US Centers for Disease Control and Prevention (CDC). Category A agents are defined by the CDC as those that "... result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption and require special action for public health preparedness".

While there are currently seven known antigenic serotypes of BoNT, only serotypes A, B, and E are predominantly associated with human intoxication. Intoxication by BoNTs leads to bilateral flaccid paralysis, involving skeletal muscle and structures innervated by autonomic fibers (Habermann and Dreyer, 1986; Simpson, 1986; Shapiro *et al.*, 1998). Death is inevitable if left untreated. The toxicity of BoNTs leading to flaccid paralysis of skeletal muscle is due to their ability to block acetylcholine (ACh) release from peripheral cholinergic nerve endings. Paralysis could persist for weeks to months depending on the serotype, and the available treatment consists of supportive care including fluids, total parenteral nutrition (TPN), and mechanical ventilation. Death occurs when the diaphragm and intercostal muscles become sufficiently compromised to impair ventilation or when patients succumb to secondary infections following long periods of intensive care (Hatheway *et al.*, 1984; Shapiro *et al.*, 1998; Robinson and Nahata, 2003).

BoNTs pose a serious concern to our national security. The toxins are highly lethal, easy to isolate, and easy to deliver by terrorists. Activities of hostile nations, international terrorists, and antigovernment groups make the threat of BoNTs a serious problem for both our military and civilian populations. Development of BoNTs as weapons of mass destruction began over 50 years ago. Nonstate-sponsored terrorists have an interest in BoNTs; members from the Aum Shinrikyo cult attempted to disperse BoNT in Tokyo and at US military installations throughout Japan on at least three occasions in the 1990s (Brackett, 1996). Furthermore, in the years following Operation Desert Storm (1990–1991), it was discovered that Iraq produced thousands of liters of concentrated BoNT in their weapons program. Moreover, approximately half of that volume was already loaded into military weapons. The BoNT generated by Iraq has yet to be fully accounted for. An act by terrorists to release BoNT into a civilian population through contamination of products of consumption or inhalation would pose a serious threat to national security and public safety. Wein and Liu (2005) modeled the threat of deliberate BoNT release into a milk-processing facility. The actions by such a terrorist group could lead to massive casualties. Another study estimated that dispersal of BoNT via inhalation could kill 10% of an exposed population within 0.5 km downwind of the incident.

II. BACKGROUND

A. Toxin Structure and Molecular Function

1. Background

The highly potent neurotoxins synthesized by the *Clostridium botulinum* microorganism and several related clostridial species (*C. baratii*, *C. butyricum*, and *C. argentinense*) are the causative agents of botulism, a potentially lethal disease historically associated with the ingestion of contaminated food products. Seven different BoNTs, designated A through G, are currently known to be produced by various strains of clostridial bacteria; these neurotoxins are antigenically distinct but comparable in basic structure. The BoNTs are members of a superfamily of homologous proteins that also include tetanus neurotoxin. BoNTs are generated as single-chain polypeptides which are then post-translationally modified (proteolytically nicked) to yield a disulfide bond-linked dichain structure composed of a heavy chain (H-chain or HC) and a light chain (L-chain or LC). Enzymes synthesized by these microorganisms themselves often mediate this cleavage although the gastrointestinal enzymes of the host can also generate the dichain structure from the ingested toxin. The three-dimensional dichain protein structure of the purified toxin is provided (see Figure 30.1).

2. Function of Heavy and Light Chains

The HC and LC of BoNTs each play critical roles in toxicity. HC is thought to mediate binding and internalization of the toxin at peripheral nerve synapses. LC, the toxic moiety, inhibits neurotransmitter exocytosis through its zinc-dependent endoproteolytic activity. The LCs of the various BoNT serotypes differ in their distinct molecular targets within the peripheral cholinergic nerve terminals (Schiavo *et al.*, 1992, 1993a, b, 1994; Blasi *et al.*, 1993; Yamasaki *et al.*, 1994). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects, despite their distinct targets.

BoNT serotype A is the most well characterized of the different serotypes in terms of both structure and function.

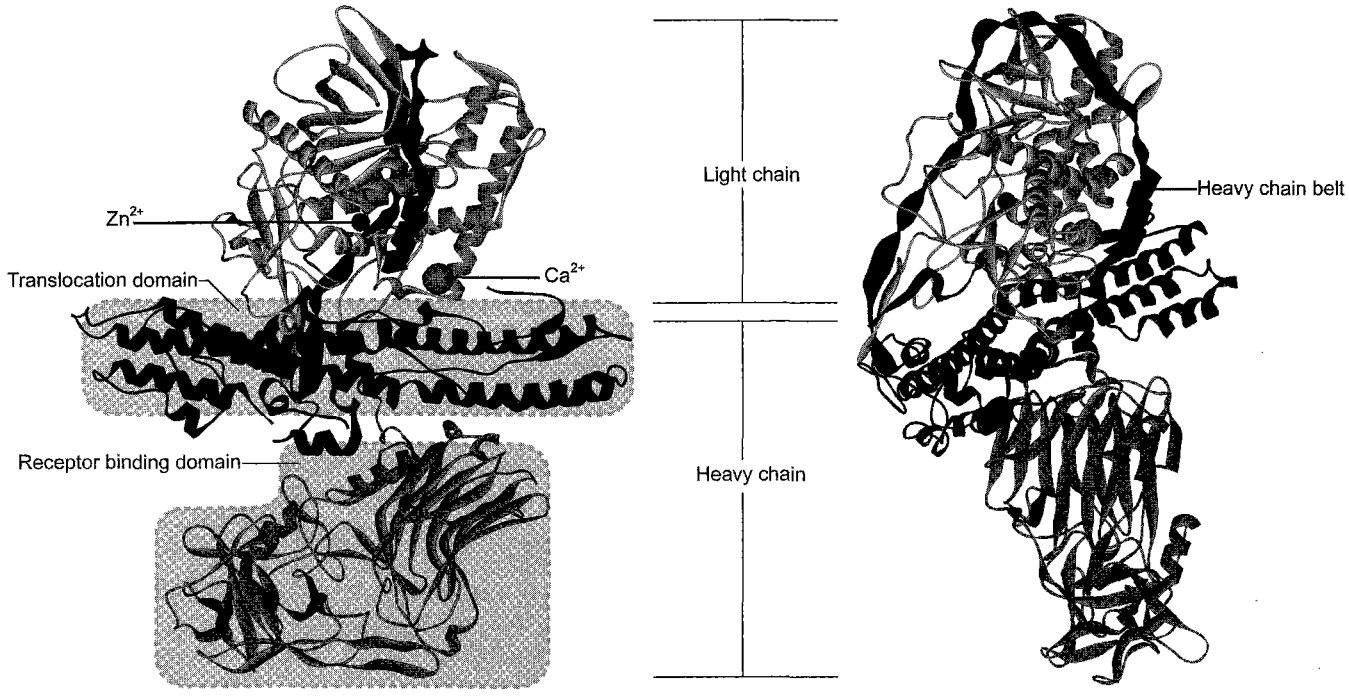

FIGURE 30.1. Three-dimensional structure of botulinum toxin serotype A (BoNT/A). BoNT/A (1,296 amino acids), rendered as a ribbon structure, is depicted in two views. BoNTs are synthesized as a single polypeptide and nicked by bacterial proteases to form a dichain molecule. The 50 kDa light chain (LC), 448 residues, and the 100 kDa heavy chain (HC), 848 residues, are linked by a dilsulfide bond. All BoNTs comprise three major domains: a receptor binding domain (C-terminal end of HC), a translocation domain (N-terminal end of HC), and a zinc-binding metalloprotease domain on LC. All seven BoNTs exhibit conserved sequence, but are also antigenically distinct at the same time. The LC seems to be held in place by the translocation belt of HC (Brunger *et al.*, 2007). The belt spans residues 492–545 in BoNT/A and 481–532 for BoNT/B and wraps around the catalytic domain of LC. Brunger *et al.* (2007) suggest that the belt acts as a surrogate pseudosubstrate inhibitor of the LC protease and acts as a chaperone during translocation across the endosome membrane into the cytosol. The belt occludes access to the active site of LC, thereby holding the unreduced holotoxin in its catalytically inactive state. The sphere represents the bound Zn^{2+} at the LC active site. The structure of BoNT/A holotoxin was provided free of copyright restrictions from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (Berman *et al.*, 2000; PDB ID: 2nz9; Garcia-Rodriguez *et al.*, 2007) and rendered using Accelrys DS Visualizer 2.0 software.

Early biochemical efforts led to its crystallization, and this crystalline form was used in numerous animal studies on the pathogenesis of botulism. Crystalline type A toxin has a sedimentation constant of 19S (around 900 kDa), which is far larger than the combined size of the HC and LC components (Simpson, 1981). This large "progenitor toxin" form was shown to dissociate under moderately alkaline conditions, releasing the "derivative" (7S) or neurotoxin component (Heckly *et al.*, 1960; Sugii *et al.*, 1977a, b; Chen *et al.*, 1998). The derivative neurotoxin has a molecular weight of 150 to 160 kDa, representing the combined size of the heavy and light chains. Additional work led to the finding that the dichain molecules comprising the various neurotoxins are often released as higher-order polypeptide complexes. The crystalline type A progenitor toxin consists of the 7S neurotoxin and one or more noncovalently linked accessory proteins. These nontoxic components of the progenitor toxin complex were later identified as three different hemagglutinins (HAs) and a nontoxic nonhemagglutinin (NTNH) protein.

3. Accessory Proteins of the Progenitor Toxin Complex

The accessory proteins of the progenitor toxin serve to enhance the stability of the toxin to ensure uptake from the gut. The NTNH component of the multimeric type A toxin complex is encoded by a single gene upstream of the neurotoxin locus while three different HA proteins have been characterized in association with BoNTs. All *C. botulinum* serotypes have been shown to produce neurotoxin complexes with the NTNH and HA proteins.

B. Overview of BoNT Action

After gaining entry to the lymphatics and circulation via the gastrointestinal tract, BoNTs function as potent neuromuscular blockers. The cellular and molecular mechanisms involved in toxin absorption, transit to specific target tissues, escape from the vasculature, and uptake within peripheral cholinergic nerve terminals have yet to be fully characterized. However, each of the neurotoxins has been shown to block vesicular neurotransmitter release at peripheral cholinergic synapse through the endoproteolytic cleaveage of proteins associated with the exocytosis machinery (Schiavo *et al.*, 1995, 1993a, b; Blasi *et al.*, 1993). At peripheral cholinergic nerve endings, BoNT binding to high-affinity receptor(s) leads to acceptor-mediated endocytosis and low pH-induced translocation across the endosomal membrane into the cytosol. The carboxy-terminal region of the toxin heavy chain appears to mediate binding at the nerve synapse while the amino-terminal domain controls translocation. The LC is held in close association with the HC by an amino acid belt. The LC of each toxin functions as a zinc-dependent endoprotease, cleaving at least one of three soluble *N*-ethylmaleimide-sensitive fusion (NSF) protein attachment receptor (SNARE) proteins involved in neurotransmitter release.

Stimulus-evoked, calcium-dependent release of acetylcholine (ACh) from the cholinergic synapse normally occurs through the formation of a fusion complex between ACh-containing vesicles and the intracellular leaflet of the nerve terminal membrane (Arnon *et al.*, 2001). This synaptic vesicle fusion complex consists of several proteins of the SNARE family, including a 25 kDa synaptosomal associated protein (SNAP-25), vesicle-associated membrane protein (VAMP, or synaptobrevin), and the synaptic membrane protein syntaxin. Other SNARE proteins have been identified as components of membrane transport systems in yeast and mammals but have not been implicated as targets for BoNTs. Meanwhile, type A and E neurotoxins cleave SNAP-25 while types B, D, F, and G act on VAMP and type C1 toxin cleaves both syntaxin and SNAP-25. Neurotoxin-mediated cleavage of any of these substrates disrupts the processes involved in the exocytotic release of ACh and leads to flaccid paralysis of the affected skeletal muscles.

C. Clinical Forms of Botulism in Humans and Animals

Exposure to BoNTs can produce lethal disease in humans and other animal species. Six different clinical forms of human botulism have been described in the literature (see Table 30.1). These include: (1) foodborne botulism, (2) infant botulism, (3) wound botulism, (4) an adult form of infant botulism, (5) inadvertent systemic botulism, and (6) inhalation botulism (reviewed by Cherington, 1998; Middlebrook and Franz, 2000; Arnon *et al.*, 2001). Botulism is the result of either an infectious process, involving elaboration of toxin from the colonizing clostridial organism, or a noninfectious process. Infant and wound botulism are the most prevalent infectious forms of botulism. Although rare, an adult form of infant botulism has been documented; gastrointestinal colonization in adults may be enabled by alterations in normal gastrointestinal flora resulting from antibiotic treatment (Cherington, 1998). Foodborne and inhalational have noninfectious etiologies and are the result of ingesting or inhaling preformed toxin. Although only one inhalational botulism incident has been reported in humans, this incident demonstrates that humans are susceptible to respiratory intoxication similar to that which has been experimentally produced in many laboratory species (Holzer, 1962; reviewed by Middlebrook and Franz, 2000). Finally, the emergence of a multitude of therapeutic applications for BoNTs has led to infrequent cases of inadvertent systemic botulism resulting from local toxin injection (reviewed by Arnon *et al.*, 2001).

D. Infectious Forms of Botulism

1. Infant Botulism

Infectious botulism is a consequence of ingesting or inhaling clostridial spores which colonize the large intestines,

TABLE 30.1. Clinical forms of botulism

	Infectious			Noninfectious		
	Infant	**Wound**	**Adult colonization**	**Foodborne**	**Inhalational**	**Inadvertent systemic**
Cause	Colonization of immature intestinal tract	Wound colonization resulting from contact with contaminated material	Intestinal colonization secondary to disruption of normal intestinal flora	Ingestion of preformed toxin in contaminated food products	Respiratory exposure to toxin aerosols or droplets	Systemic toxin uptake after therapeutic toxin administration
Susceptibility	Young infants (2 to 4 months of age) prior to establishment of normal intestinal flora	Self-administering users of intravenous drugs (often black tar heroin)	Antibiotic-treated patients	All exposed individuals	All exposed individuals	Patients treated with local toxin injections

germinate, and elaborate toxin into the bloodstream. Infant and wound botulism are the most prevalent infectious forms of botulism. Infant botulism comprises the majority (72%) of reported human botulism cases in the USA, while most of the remaining cases involve foodborne and wound botulism (Mackle *et al.*, 2001). Young infants are especially susceptible to infant botulism. Infants were found to be uniquely susceptible to gastrointestinal colonization due to a lack of well-established competing gut flora (Arnon, 1995). While infant botulism can be acquired by inhalation of spores, this differs markedly from inhalational botulism, which results from inhaling preformed aerosolized toxin and not spores. Clostridial spores do not pose a threat in older infants or most adults (Arnon, 1995, 1998; Cox and Hinkle, 2002).

2. Wound Botulism

Wound botulism involves growth of *C. botulinum* spores in a contaminated wound with *in vivo* toxin production (Weber *et al.*, 1993). It accounts for less than 25% of all botulism cases (Sandrock and Murin, 2001). The majority of wound botulism cases are caused by serotype A and the remainder by serotype B (Shapiro *et al.*, 1998). The neurological symptoms of wound botulism differ little from those of foodborne botulism except for the general absence of gastrointestinal symptoms. From its discovery in 1943 until 1996, only 111 incidents of wound botulism were documented (CDC, 1998; Merson and Dowell, 1973; Shapiro *et al.*, 1998); among the 100 laboratory confirmed cases, 83 cases were type A, 16 cases type B, and one a mixture of type A- and B-producing organisms (Hatheway, 1988; CDC, 1998). Risk factors for wound botulism include deep wounds, avascular areas, compound fractures, and crush injuries of the hand. Although a rare form of naturally occurring BoNT intoxication, it most recently occurred in Maryland as a result of a construction worker receiving a contaminated, compound fracture of the femur after falling into an excavated pit (Hilmas, personal observation). Wound botulism also occurs in intravenous drug users as a result of bacterial colonization at needle puncture sites or nasal/sinus lesions secondary to cocaine snorting (MacDonald *et al.*, 1985). From 1986 through 1996, 78 cases of wound botulism were reported, and the majority of cases were linked to black tar heroin, introduced intravenously.

3. Child or Adult Botulism from Intestinal Colonization

Gastrointestinal colonization in adults or children by clostridial bacteria does not typically take place except under circumstances where the normal flora has been altered by antibiotic treatment (Cherington, 1998). Botulism results from *in vivo* production of toxin, analogous to the pathogenesis of infant botulism (McCroskey and Hatheway, 1988; Chia *et al.*, 1986). Support for this form of botulism is provided by demonstration of prolonged excretion of toxin and *C. botulinum* in stool and/or by the demonstration of *C. botulinum* spores but not preformed toxin in suspected foods.

E. Noninfectious Forms of Botulism

1. Foodborne Botulism

Worldwide, BoNT intoxication is most commonly associated with food poisoning. In the early 19th century, the effects of botulism were observed to be associated with the consumption and handling of meat products. Thus German physician Justinus Kerner described what he termed "sausage poisoning" (Erbguth, 2004). It was later in the 19th century that the term "botulism" was used, from the Latin *botulus* for "sausage". Foodborne botulism results from ingesting preformed toxin in food contaminated with toxin spores. Inadvertent and inhalational botulism, two other noninfectious forms of botulism, also involve

exposure to preformed toxin. Outbreaks of foodborne botulism in the USA result from eating improperly preserved home-canned foods (CDC, 1995). The majority of cases of foodborne botulism are due to serotypes A, B, and E. From 1990 through 1996, type A accounted for 44.6% of foodborne outbreaks in the USA, followed by type E (35.7%) and type B (12.5%) (CDC, 1998). The prompt recognition of such outbreaks in the USA and early treatment with serotype-specific botulinum antitoxin has limited the number of casualties, severity of the disease, and the case-to-fatality ratio. Mortality from foodborne botulism has declined from 60% (CDC, 1998) in 1950 to less than 10% of clinical cases (Shapiro *et al.*, 1998).

2. Inhalational

Because humans are relatively resistant to gut colonization by the *C. botulinum* microorganism, oral and inhalational exposures to preformed neurotoxin are likely to present the greatest threats with respect to intentional dissemination. The ability for inhaled botulinum neurotoxins to produce illness has been documented in humans and in several experimental species. Only one incident involving inhalational intoxication in humans has been reported. Three laboratory workers presented with physical and neurological symptoms after accidental respiratory exposure to aerosolized type A toxin (Holzer, 1962; Middlebrook and Franz, 2000; Arnon *et al.*, 2001). These patients were all successfully treated with antiserum, gradually recovering from their weakness and visual disturbances over the next several days. After inhalational exposure, the neurotoxins are absorbed from the respiratory tract into the lymphatics and circulation for transport to peripheral cholinergic synapses (reviewed by Simpson, 2004). The pathogenesis following neurotoxin absorption is thought to be similar for both the respiratory and gastrointestinal exposure routes. Thus, the primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism.

3. Inadvertent Systemic Botulism

The therapeutic indications for BoNTs are numerous. They are used in the treatment of ophthalmological disorders (strabismus, Duane's syndrome, esotropia/exotropia), movement disorders (focal dystonias, blepharospasm), spasticity, neuromuscular disorders, pain (headache, myofacial pain), disorders of the pelvic floor (anal fissures), ear/nose/throat disorders, cosmetic applications (wrinkles), and hyperhidrosis. The recent explosion in new indications for BoNTs in the treatment of a wide range of medical conditions also brings the possibility for medical errors in BoNT dosing. Systemic botulism may result from injection of excessive doses of the potent neurotoxin. The most infamous case of systemic botulism involved the paralysis of four Florida patients, including the doctor, treated with BoNTs for wrinkles. The physician used non-FDA approved formulations of type A from Toxin Research International, Inc. The research grade type A neurotoxin was apparently sold to the doctor and reconstituted to be thousands of times more potent than the typical dose used in BOTOX® for paralyzing facial muscles. Later testing estimated that the raw bulk toxin used contained between 20,000 and 10 million units of botulinum toxin. In comparison, a typical vial of BOTOX from Allergan, Inc. contains only 100 units (CIDRAP, 2004). All three patients and the physician were injected with the toxin preparation; they developed severe systemic botulism requiring mechanical ventilation. While all four survived the super-lethal dose of type A toxin, several of the patients have experienced a syndrome involving chronic gastrointestinal symptoms and discomfort months after exposure.

F. Human Intoxication

The basic syndrome of BoNT intoxication is similar for all naturally occurring forms, as well as for inhalation exposure and does not vary appreciably among serotypes (Simpson, 1986; Habermann and Dreyer, 1986; Hatheway *et al.*, 1984; Jankovic and Brin, 1997). Based upon documented laboratory evidence, human BoNT intoxication is caused by exposure primarily to serotypes A, B, E, and to a much lesser extent to serotype F; disease manifests mostly as a result of foodborne, infant, and wound botulism (Habermann and Dreyer, 1986; Simpson, 1986). BoNTs are also lethal from inhalation of aerosolized toxin, although this form is not generally observed in nature.

The various toxin serotypes are usually associated with analogous clinical presentations. Paralysis proceeds in a descending fashion after an initial bulbar involvement. The earliest symptoms of botulism typically include visual disturbances, followed by dysphagia, dysphonia, and dysarthria, reflecting an especially high susceptibility of cranial efferent terminals to BoNT action (Habermann and Dreyer, 1986; Jankovic and Brin, 1997). A descending generalized skeletal muscle weakness may then develop, progressing from the upper to lower extremities. Involvement of the diaphragm and intercostal muscles can lead to ventilatory failure and death unless appropriate supportive care is provided (Shapiro *et al.*, 1998; Robinson and Nahata, 2003).

III. EPIDEMIOLOGY

A. Foodborne Botulism

Human foodborne botulism outbreaks have typically been linked to the consumption of toxin-contaminated home-prepared or home-preserved foods (Maselli, 1998). The vast majority of foodborne botulism cases are attributed to toxin types A, B, or E. Maselli (1998) reports that type B is the most prevalent (52%) in the USA, followed by type A (34%) and type E (12%), while the CDC (1998) suggests 37.6% of all foodborne botulism outbreaks since 1950 were caused by

type A, 13.7% by type B, 15.1% by type E, 0.7% by type F, and 32.9% were unidentified with respect to toxin type. Outbreaks of type F and G botulism are rare (Sonnabend *et al.*, 1981; Maselli, 1998; Richardson *et al.*, 2004), and only anecdotal reports of isolated type C1 and D botulism cases can be found in the published literature (Lamanna, 1959).

The natural epidemiology of foodborne botulism provides additional insight into the similarities and discrepancies between the human disease and that represented in various animal models. In the USA, around 25% of reported human botulism cases are classified as foodborne and 72% are infant (Mackle *et al.*, 2001). Human type A and B foodborne botulism cases occur worldwide and constitute the vast majority of reported human intoxications (Maselli, 1998). The majority of other botulism cases are attributed to serotype E and are typically associated with the consumption of contaminated seafood. Generalizations have been made regarding the geographic distribution of the most common *C. botulinum* strains within the USA. Most human foodborne botulism outbreaks occurring west of the Mississippi are due to type A toxin; type B strains are more prevalent east of the Mississippi while type E strains are typically isolated to Alaska and the Pacific Northwest (Arnon *et al.*, 2001; Richardson *et al.*, 2004).

Several clinical and epidemiological reports have evaluated the worldwide geographic distributions of human foodborne botulism cases. A review of 13 outbreaks between 1970 and 1984 identified geographic differences in the toxin serotypes associated with human foodborne botulism cases. Type B botulism was predominant in Portugal, Spain, France, and several other European countries (Lecour *et al.*, 1988). Interestingly, the low mortality rate associated with human type B foodborne botulism (8.8% versus 24.3% for type A and 30.8% for type E in the USA from 1950 to 1979) did not correlate with the high oral toxicity for type B toxin in mice (Ohishi *et al.*, 1977; Sugii *et al.*, 1977a, b, c; Ohishi, 1984). Serotype E was linked to botulism outbreaks in select regions such as the Baltic countries (Lecour *et al.*, 1988) and typically resulted from the consumption of contaminated fish (Maselli, 1998).

Type F toxin was only associated with two reported outbreaks of human foodborne botulism prior to 1998 (Maselli, 1998). The first of these outbreaks occurred in Denmark (on the Island of Langeland) and was attributed to a contaminated liver paste product (Muller and Scheibel, 1960; Richardson *et al.*, 2004). The second outbreak, in 1966, affected three individuals in California and was associated with home-made venison jerky (Midura *et al.*, 1972; Richardson *et al.*, 2004). While a few other type F botulism cases have been reported, they are generally thought to have resulted from intestinal colonization and type F toxin production by another related species, *C. baratii* (Hall *et al.*, 1985; Richardson *et al.*, 2004). A recent report of a type F botulism case in California provided some additional insight into this uncommon toxin serotype and the associated clinical disease (Richardson *et al.*, 2004). The patient described in this report presented with typical signs and symptoms including ptosis, dysphagia, and weak extremities. Although the source of the ingested toxin was not conclusively determined, the exposure was tentatively linked to shellfish consumption, and type F toxin was subsequently detected in the patient's stool (Richardson *et al.*, 2004). Human type F botulism cases may have been underreported in the past since some laboratories did not test culture isolates for the presence of *C. baratii*, which also produces type F toxin.

Type G toxin-producing clostridial organisms (*C. argentinense*) have been detected in several soil samples from a South American cornfield (Gimenez and Cicarelli, 1970; Maselli, 1998), but only one reported outbreak of type G botulism (in Switzerland) has been identified in the published literature (Sonnabend *et al.*, 1981). Certain aspects of this outbreak draw questions as to whether it was truly associated with type G intoxication. Type G organisms were isolated from all four affected adults and an 18-week-old infant, suggesting that the intoxications were due to ingestion and subsequent colonization by type G spores (Sonnabend *et al.*, 1981). Type G toxin was detected at low levels of 2–7 mouse intraperitoneal lethal dose 50 ($MIPLD_{50}$)/ml in the serum of three out of the four lethally intoxicated adults, all of whom died suddenly sometime after the presumed foodborne intoxication. Type A toxin was also detected in two of these individuals, suggesting that the intoxications may have involved colonization either by a mixed set of clostridia or by a unique strain producing multiple toxins (Sonnabend *et al.*, 1981). Alternatively, detection of dual serotypes could have been an artifact of the culture or testing methods. Soil samples taken from the area indicated the presence of only type A clostridial organisms (Sonnabend *et al.*, 1981). Regardless, the occurrence of human type G botulism is rare, and the relative susceptibility of humans to colonization and intoxication from this serotype is not clear.

Species-specific patterns of susceptibility to different toxin types are common in both naturally occurring and experimental foodborne botulism. These differences do not necessarily facilitate identification of the most appropriate animal models from the human condition, but they may help to eliminate highly variant species. For example, mink were reported to be relatively resistant to toxin types A, B, and E (Yndestad and Loftsgard, 1970), which are responsible for the vast majority of human botulism outbreaks (Maselli, 1998; Arnon *et al.*, 2001). Weanling pigs, on the other hand, were shown to be moderately resistant to types A, C1, E, and F and highly resistant to type D toxin (Smith *et al.*, 1971). Experimental and epidemiological studies have identified one persistent difference in the epidemiology of botulism in humans compared to many other animal species. Few reports citing human outbreaks of type C and D are available. One of these reports mentions two human type C outbreaks and one type D outbreak but provides no

reference for these cases (Lamanna, 1959). More recent reports of human type C or D botulism have not been found in the literature, and it is widely assumed that human foodborne intoxications are rarely, if ever, associated with these toxin types. In contrast, naturally occurring botulism of both types is quite common among domestic and wild animal species, and several studies have established the susceptibility of various laboratory species to experimental type C and D botulism.

The authors of this chapter have studied the effect of C and D toxin serotypes, as well as A, B, and E, on human intercostal muscle (Hilmas, unpublished data). All serotypes showed a similar ability to produce complete muscular paralysis in *ex vivo* human intercostal muscle. Intercostal muscle was excised from patients receiving a thoracotomy and intercostal muscle flap procedure. The muscle was removed tendon to tendon by surgical excision without electrocautery and dissected into multiple bundles with their associated intercostal nerves. The nerve–muscle units were placed in a vertical twitch bath and stimulated at 0.03 Hz (0.2 ms pulses of supramaximal strength) using a novel nerve clamp electrode to illicit an indirect muscle twitch. Potent toxins (1 nM) from various serotypes were added to the bath after confirming the stability of control muscle responses. In each case, twitch tensions declined to negligible amplitudes by 1 h after direct toxin application to the tissue bath.

Several nonhuman primate species are known to be susceptible to type C1 and D toxins both in nature and as experimental models. A large natural outbreak of type C botulism was reported in a troop of captive *hamadryas* baboons in 1989 (Lewis *et al.*, 1990). The outbreak resulted in the deaths of 36 animals, including 3 adult males, 6 sub-adult males, 17 adult females, and 10 sub-adult females. Additional animals displayed mild to moderate symptoms that resolved over a period of several days (Lewis *et al.*, 1990). As with human foodborne botulism, various age groups and both sexes were affected, and no macroscopic lesions were apparent. Serum samples and gastric contents taken from ill animals contained type C1 toxin, although the source of the toxin was not identified (Lewis *et al.*, 1990). The authors speculated that man is probably also susceptible to type C1 toxin. The reason for the relative lack of human type C botulism cases remains unknown. It has been suggested that serotype C is often associated with carrion, providing a possible explanation for the absence of reported human cases. At least two other outbreaks of naturally occurring type C botulism in nonhuman primates were previously reported, one in squirrel monkeys (*Saimiri sciureus*) and capuchin monkeys (*Cebus capucinus* and *Cebus olivaceus*) (Smart *et al.*, 1980) and the other in gibbons (*Hylobates lar*) (Smith *et al.*, 1985).

In addition to nonhuman primates, most other animal species that show some sensitivity to botulinum intoxication are in fact susceptible to toxin serotypes C1 and D. Several rodent species are susceptible to oral intoxication with most botulinum toxins, including types C1 and D (Matveev, 1959; Jemski, 1961a, b; Cardella *et al.*, 1963; Sergeyeva, 1966; Sugiyama *et al.*, 1974; Smith, 1986; Fujinaga *et al.*, 1997; Gelzleichter *et al.* 1999; Middlebrook and Franz, 2000). The majority of botulism outbreaks in cattle have also been attributed to toxins C1 and D (Schocken-Iturrino *et al.*, 1990). Cattle intoxication is typically associated with the ingestion of contaminated bones and other carcass remains; their apparent susceptibility to type C and D botulism might simply be due to frequent ingestion of decaying material that is primarily contaminated with these toxin types. A recent study indicated that cows are also uniquely sensitive to intravenous injection of type C1 toxin (Moeller *et al.*, 2003). The high susceptibility of cattle to type C botulism is not dependent on exposure route although the specific factors contributing to their sensitivity are not known.

A recent outbreak of type C botulism among farmed mink and foxes in Finland underscores the need to consider not only the quantitative susceptibility of various species to the toxins but also the potential epidemiological significance of interspecies differences in dietary patterns. Lindstrom *et al.* (2004) reviewed the Finland incident, which was the largest documented type C botulism outbreak in fur production animals. Over 52,000 animals developed illness after consumption of feed product that was contaminated with over 600 MLD of type C1 toxin per gram. This feed consisted of acidified slaughter by-products from poultry, beef, and fish (Lindstrom *et al.*, 2004). According to national regulations, these by-products were acidified with an organic acid to yield a final pH of 4.0 or lower. Such processing would inhibit the growth of many microorganisms but would not necessarily result in significant toxin inactivation. Over 44,000 of the 52,000 affected animals died, and the death rate among all potentially exposed animals was almost 22% (Lindstrom *et al.*, 2004).

The large number of animals affected and the high lethality associated with the outbreak could be considered indicative of the high susceptibility of the affected species to foodborne type C botulism. This high susceptibility might appear to be in stark contrast to that of man due to the scarcity of human type C cases. However, the Finland outbreak provides a clear indication that dietary differences between species may play a significant role in these epidemiological patterns. Humans would be far less likely to consume slaughter by-products (including intestinal tissues) as opposed to the higher quality beef, poultry, and fish products. Moreover, preparation of such products for human consumption would generally involve cooking rather than acidification. Thus, the influence of dietary habits must be taken into consideration when evaluating interspecies differences in epidemiological patterns for the various toxin serotypes.

It remains possible that humans generally do not consume the types of foods that are typically subject to contamination with type C1 and D toxins. Some researchers continue to speculate that humans are likely to be susceptible to both serotypes because they lead to botulism in monkeys both in nature and after experimental oral

exposure. Alternatively, humans might display a unique pattern of physiological susceptibility to the different toxin types. Lack of human susceptibility to type C1 and D intoxication could be attributed either to poor absorption of these specific toxins from the human gastrointestinal tract or to resistance of human cholinergic nerve terminals to the activity of these toxins. One cell culture study provided some support for the latter explanation. Type C1 neurotoxin was shown to bind with high efficiency to mouse neuroblastoma cells and to hybridomas of mouse neuroblastomas and rat gliomas, but not to human neuroblastoma cell lines (Yokosawa *et al.*, 1989). Yokosawa *et al.* (1989) suggested that reduced binding of type C1 toxin to human versus mouse neuroblastoma cells could provide one explanation for the lack of human type C botulism cases.

Another potential explanation for the unique epidemiology of human botulism was provided in a study of botulinum toxin binding and transcytosis across polarized monolayers of two human colon carcinoma cell lines (T-84 and Caco-2). Substantial binding of iodinated BoNT/A and BoNT/B to human colon carcinoma cells was observed while minimal binding of type C1 neurotoxin was detected (Maksymowych and Simpson, 1998). Both type A and B neurotoxins were also efficiently taken up, transcytosed, and released, by the polarized human carcinoma cells, whereas minimal transcytosis of type C1 neurotoxin was observed. The patterns of neurotoxin transcytosis (A and B but not C1) observed in these human gut epithelial cell lines correlate with human susceptibility to foodborne botulism (Maksymowych and Simpson, 1998). The authors speculated that since human tissues are fully sensitive to the neuromuscular blocking properties of C1 neurotoxin (Coffield *et al.*, 1997; Eleopra *et al.*, 2004), the relative absence of human foodborne type C botulism could be due to the inability of this toxin to penetrate from the gut to the general circulation. Human susceptibility to type C1 and D neurotoxins remains unclear; however, clarification of this issue will be important in interpreting data derived both from *in vitro* studies on toxin transcytosis and from animal models for oral intoxication.

IV. PATHOGENESIS

A. Overview of Pathogenesis

BoNTs are a group of immunologically distinct but closely related bacterial proteins that act as potent inhibitors of synaptic transmission in skeletal muscle. Inhibition of ACh release from the presynaptic terminal of the neuromuscular junction (NMJ) is thought to be the sole mechanism involved in the toxins' lethal action (Sellin, 1985; Simpson, 1986) and therefore the cause of botulism. The pathogenesis of intoxication is not completely understood but is generally thought to involve a multistep process to interrupt normal vesicular release of ACh from the presynaptic motor nerve terminal. In a process of transcytosis, ingested or inhaled BoNT must first cross a barrier (either intestinal or pulmonary epithelial cells) to gain access to the circulation (see intestinal absorption of BoNT, Figure 30.2). Once in the circulation, BoNT travels to its major target, located on presynaptic membranes of alpha motor neurons at NMJs and neuroeffector junctions. Toxin binding through its heavy chain to a cell surface receptor on the presynaptic motor nerve ending is followed by internalization via an endocytotic vesicle, acidification of the endosome, conformational change allowing cleavage of the enzymatically active light chain (LC) from bound heavy chain (HC), and release of light chain toxin into the cytoplasm. Here the light chain cleaves one of the integral members of the SNARE complex (SNAP-25, VAMP, or syntaxin), proteins involved in exocytosis of ACh (Simpson, 1986, 2004; Black and Dolly, 1986; Blasi *et al.*, 1993; Montecucco *et al.*, 1994; Schiavo *et al.*, 1995). BoNT thereby prevents docking of synaptic vesicles with presynaptic plasma membrane by selective proteolysis of synaptic proteins. Each stage in BoNT action provides a potential point for pharmacological intervention.

B. Toxin Stability

1. Biological Stability of the Toxins in the Gastrointestinal Tract

A major factor to consider in botulism is the stability of both the organism and the toxin. A variety of factors can affect the stability of ingested BoNTs within the gastrointestinal tract. The oral potency of the toxins is closely related to their ability to withstand the conditions found in these biological compartments prior to absorption into the lymphatics and general circulation. The stability of BoNT preparations has therefore been examined in the gastrointestinal compartments of intoxicated animals, as well as under different enzymatic and acidic conditions *in vitro*. Some of the earliest work on BoNT intoxication indicated that the stability and resulting potency of type A toxin vary both qualitatively and quantitatively in different rodent species (Minervin and Morgunov, 1941).

Several groups have evaluated the influence of ingested foods on the gastrointestinal stability and potency of BoNTs. Lamanna and Meyers (1959) reported that the ingestion of protein- or fat-containing foods prior to oral type A exposure in mice resulted in moderate increase in toxicity. The mechanisms by which food intake enhanced toxin potency were not clarified; however, the relatively small observed increases (two-fold) could have been to normal experimental variation in determining oral toxicity values. The same study demonstrated that fluorescein-labeled type A toxin was quickly destroyed in the stomach of mice. Crystalline and purified toxins form stable complexes with albumin and other proteins found in food and serum (Lamanna and Meyers, 1959). Albumin was later shown to prevent loss of potency when type A toxin was

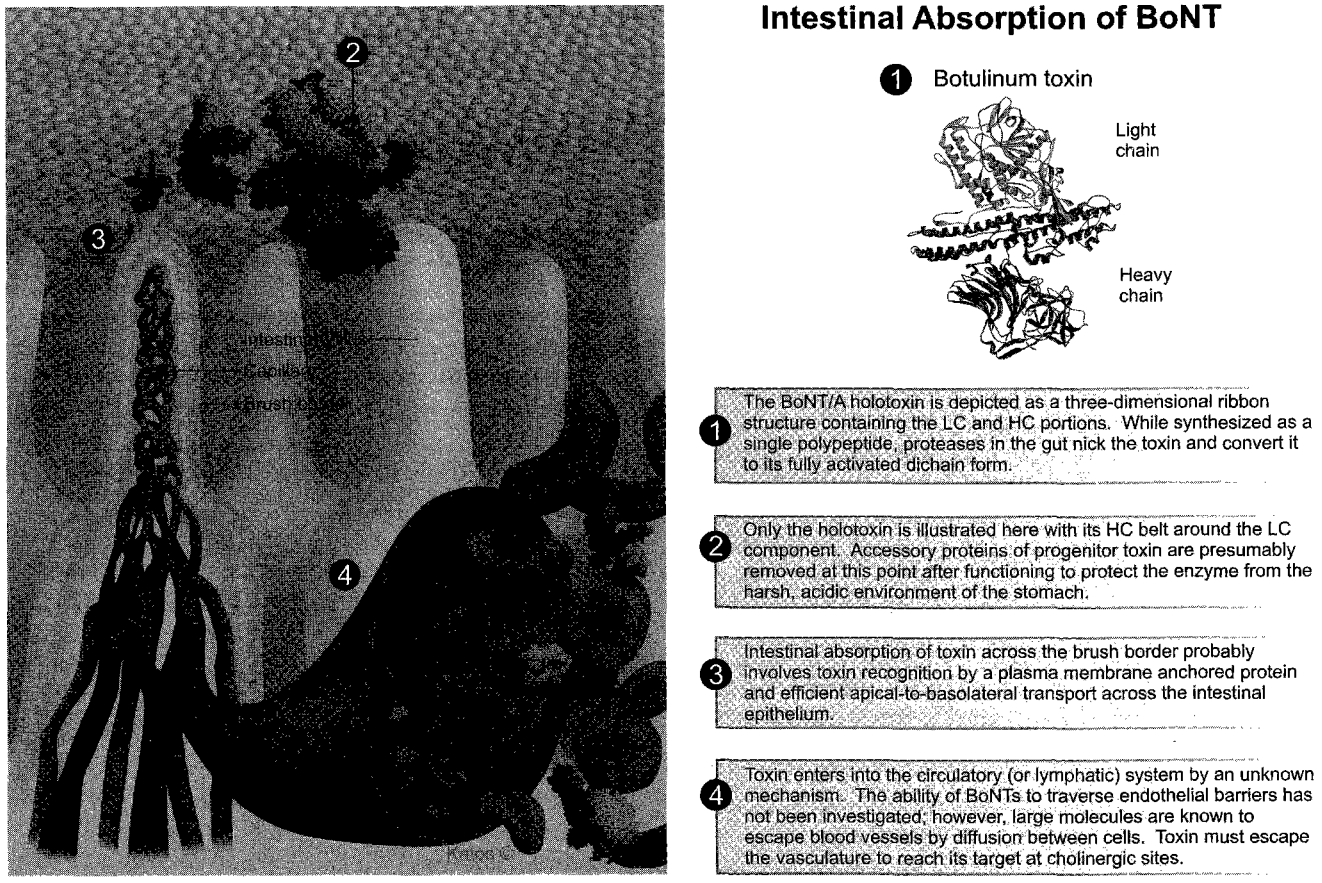

FIGURE 30.2. Intestinal absorption of botulinum neurotoxin. Neurotoxin present in the gut lumen as a result of foodborne (ingested toxin) or infant botulism (toxin synthesized by clostridial spores) must cross the epithelial membrane to reach the circulatory and lymphatic systems. The toxin presumably binds to an as yet unidentified receptor on the intestinal villus epithethium that is linked to an efficient transport process. Progenitor toxin contains nontoxic HA and NTNH accessory proteins which are probably shed from the protein prior to entry across the intestine brush border. Progenitor toxin is thought to be too large for any significant rate of paracellular diffusion (Simpson, 2004). The ability of botulinum holotoxins without accessory proteins to traverse endothelial barriers has not been investigated; however, large molecules are known to escape blood vessels by diffusion between cells. Toxin escapes the circulatory system and reaches peripheral (and possibly central) cholinergic sites. These include neuromuscular junctions, ganglia of the sympathetic and parasympathetic nervous system, postganglionic parasympathetic sites, and postganglionic sympathetic sites that release ACh. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

exposed to a wide range of pH values (Zacks and Scheff, 1967). This observation was consistent with the more recent finding that the enzymatic activity of BoNT A was enhanced in the presence of albumin (Schmidt and Bostian, 1997).

Subsequent studies expanded upon this early work by investigating the stability of other toxin types and preparations in solutions having different pH values and other conditions similar to those encountered in the gastrointestinal tract. Type C1 toxin was stable in most acidic and basic environments, as significant inactivation (as indicated by loss of toxicity in a mouse lethality assay) was only observed following exposure to extreme pH values (pH 1.8 and pH 12) (Halouzka and Hubalek, 1992). Progenitor type E toxin was more stable than its derivative (purified) form, which was subject to rapid inactivation when exposed to pH values less than 4.0 (Sakaguchi and Sakaguchi, 1974). This study demonstrated that type E progenitor toxin dissociated either during or after gastrointestinal absorption in mice, as only the derivative component could be detected in the blood and lymph following oral administration of the progenitor form.

Similar findings on the relatively high stability of progenitor versus derivative forms of the other toxin serotypes have been reported in other studies. Types A, B, and F progenitor toxins were more stable under conditions of low pH, as well as more resistant to digestion by pepsin and papain, than their corresponding derivative toxins (Sugii *et al.*, 1977a, b).

The derivative forms of toxins A and B were almost completely inactivated after 10 min of peptic digestion at pH 2.0, while the progenitor forms retained over 60% toxicity after an 80 min treatment (Sugii *et al.*, 1977a). Crystalline type A toxin was shown to be partially resistant to proteolysis by trypsin, retaining 25% of the potency of

control-treated toxin even after a 72 h trypsin digestion at 37°C (Coleman, 1954). The crystalline toxin was more readily inactivated by digestion with pepsin at pH 1.4 and chymotrypsin at pH 6.5. Interestingly, another group reported that the potency of type A toxin was weakened by 80% after a 5 h incubation in phosphate buffer (pH 7.5), while toxins C1 and D maintained 100% toxicity under the same conditions (Miyazaki and Sakaguchi, 1978). These findings demonstrate both serotype- and enzyme-dependent effects on the *in vitro* stability of the BoNTs that are also likely to impact their persistence in the gastrointestinal tract. A similar pattern of stability among the various BoNT forms in gastrointestinal juices isolated from different animal species has been demonstrated (Sugii *et al.*, 1977a). The progenitor forms for all toxin serotypes retained significant toxicity in comparison to their derived holotoxin counterparts.

Epitope mapping experiments suggested that the nontoxic component of the intact progenitor toxin complex covers a large portion of the binding domain of the neurotoxin (Chen *et al.*, 1998). Toxin interaction studies also revealed that the purified neurotoxin adheres to lipid monolayers while the progenitor complex is not subject to significant adsorption to the same monolayers. This observation led to speculation that the protective nontoxic components (HA and NTNH) may also facilitate progenitor toxin transit through the gastrointestinal tract by minimizing neurotoxin adherence to lipid membranes (Chen *et al.*, 1998). On the other hand, toxicity studies suggest that adherence to lipid membranes is not critical for neurotoxin absorption since the intact progenitor complex is generally much more potent by the oral route than the purified neurotoxin. Moreover, the neurotoxin has been shown to protect the agglutination capacity of the associated nontoxic HA components within the progenitor toxin complex (Chen *et al.*, 1998).

Importantly, the nontoxic HA components of the progenitor complex appear to protect the neurotoxin from proteolysis and degradation under pH extremes while the agglutinating activity of the nontoxic component is maintained by the presence of the neurotoxin. The type A progenitor toxin complex contains several HA components that might contribute to protecting the neurotoxin from degradation. One of these HA components, referred to here as HA-33 (or HA1 in some studies), was shown to interact directly with type A neurotoxin and to significantly increase its resistance to enzymatic proteolysis *in vitro* (Sharma and Singh, 2004). The authors of this work hypothesized that HA-33 provides protection against enzymatic degradation either by blocking the accessibility of protease-sensitive sites on BoNTs or by inducing structural changes within the neurotoxin itself.

Collectively, these studies offer important insight into the relative stability of the BoNTs within the gastrointestinal tract based upon their resistance to inactivation under various enzymatic conditions. In general, toxin stability directly correlates with the presence of the accessory HA and NTNH components of the multimeric progenitor toxin complex. These proteins appear to function in protecting the neurotoxin from degradation or inactivation. The various toxin serotypes also display unique resistances to enzymatic digestion although the basis for these differences is not known.

C. Oral Intoxication: Toxin Absorption from the Gastrointestinal Tract

1. Role of Progenitor Toxin Accessory Proteins

The role of the nontoxic accessory proteins within the progenitor toxin complexes is not fully understood. They appear to function in protecting the ingested toxins from degradation and in facilitating absorption from the gastrointestinal tract. Functional characterization of the HA and NTNH proteins has been advanced by biochemical techniques for generating toxin preparations containing only select components of the progenitor complex. These 7S toxins are relatively sensitive to proteolytic degradation and denaturation in the stomach (Schiavo *et al.*, 1992). The auxiliary HA and NTNH proteins within the multimeric complex have been shown to dramatically increase the stability of the associated neurotoxin during transit through the gastrointestinal tract (Ohishi *et al.*, 1977; Fujinaga *et al.*, 1997; Sugii *et al.*, 1977a, b, c). The multimeric complex is then thought to readily dissociate either in the intestine or after absorption into the circulation. Most studies suggest that the accessory proteins do not appear to have any involvement in the activity of the toxins at peripheral nerve terminals. Thus, the HA and NTNH components are likely to be dispensable in disease pathogenesis after parenteral or respiratory exposure, where the toxins bypass the harsh conditions of the gastrointestinal tract.

2. Role of Enterocytes

Both absorptive enterocytes and Peyer's patch-associated M cells have been implicated in toxin transcytosis from the gastrointestinal tract after oral exposure. Peyer's patches are collections of lymphoid tissue that are part of the gut-associated lymphoid tissue. M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. Unpublished work by Park and Simpson (2003) indicates that knockout mice deficient in Peyer's patch M cell complexes are still susceptible to both oral and respiratory botulinum intoxication and the development of HC-specific antibody responses. Based upon these results, the authors suggest that M cells are not likely to be involved in toxin uptake and processing from the respiratory tract. In addition, both cell types have comparable transcytosis rates (M cells are five times as efficient in transcytosis than intestinal enterocytes), but enterocytes greatly outnumber M cells; therefore, gastrointestinal enterocytes are the predominant cell types involved in toxin uptake and processing from the gastrointestinal tract.

Maksymowych and Simpson (1998) used transwell culture systems with various transformed epithelial cell lines to evaluate the fate of the HA components of type A progenitor toxin complex. Radiolabeled preparations of both BoNT/A and HA were taken up by cultured T-84 human colon carcinoma cells by bulk endocytosis. However, efficient delivery across the T-84 cells was only observed for the neurotoxin.

D. Respiratory Intoxication

The potential threat posed by aerosolized botulinum toxins is emphasized by their ease of production, their extremely high potency relative to other biological toxins, and their use in various weaponization programs over the past several decades (Arnon *et al.*, 2001). This threat, along with the relative lack of information on respiratory toxicity and pathogenesis in humans, has fueled research on inhalational botulism in several animal models including mice, rabbits, guinea pigs, mongrel dogs, and rhesus monkeys.

1. Toxin Absorption from Respiratory Tract

The relative persistence and absorption of the toxins following experimental respiratory exposure have been investigated in a few animal species. An early literature review suggests that type A toxin is more potent in mice by the respiratory route than by subcutaneous (SC) administration but less potent by the intraperitoneal (IP) route (Morton, 1961).

Guinea pigs were shown to be highly sensitive to inhaled botulinum toxins when compared to other rodent species. Respiratory penetration and retention of inhaled toxin are higher in guinea pigs than mice (Lamanna, 1961). Toxin could be detected in the lungs of guinea pigs after intranasal (IN) administration of only 2 mouse lethal doses of type E toxin although detection in the blood or liver required higher doses (Sergeyeva, 1962, 1966). Guinea pigs were also reportedly more susceptible to type A toxin by inhalation than mice because shorter incubation periods were observed in guinea pigs prior to the onset of acute disease (Iakovlev, 1958).

Although inhalational botulinum intoxication was investigated in other animal species, these studies have not provided specific data on toxin absorption. The behavior of BoNTs in the respiratory tract was only recently investigated. Park and Simpson (2003) studied the properties of pure BoNT/A neurotoxin both *in vivo* and *in vitro* using mice and pulmonary cell culture models, respectively. Mean survival times were compared in mice receiving various doses of pure BoNT/A either IN or IP. Pure BoNT/A was found to be a potent intranasal poison, although the toxicity (as determined by mean survival time) associated with IP administration was somewhat higher. Mean survival times in mice were less than 100 (IP) or 600 min (IN) after administration of 0.1 μg pure toxin; 75 (IP) or 400 min (IN) for 1 μg toxin; and 120 min (IN) for 10 μg toxin (Park and Simpson, 2003). As seen with oral and parenteral routes, a linear relationship existed between the log of the intranasal dose administered and the geometric mean survival time. The HA and NTNH component of the progenitor toxin did not enhance toxicity, establishing different requirements for the stability and absorption of inhaled versus ingested toxin (Park and Simpson, 2003).

Transwell experiments were also performed to investigate BoNT/A transcytosis across a human pulmonary adenocarcinoma cell line (Calu-3), the MDCK cell line, and a primary rat alveolar epithelial cell line (Park and Simpson, 2003). Efficient BoNT/A transcytosis in both directions across polarized Calu-3 monolayers was observed, while toxin transcytosis occurred at a much lower rate across MDCK cells. These findings were in agreement with previous work demonstrating that the efficiency of BoNT/A transcytosis across MDCK monolayers was much lower than that observed across gut epithelial cells (Maksymowych and Simpson, 1998). BoNT/A transcytosis was also observed across primary rat alveolar cells, although at a slightly slower rate than that seen for the human adenocarcinoma cells (Park and Simpson, 2003). While the light chain of BoNT/A was not essential for transcytosis, heavy chain apical–basolateral (A-B) and basolateral–apical (B-A) transcytosis rates were somewhat lower than those of intact BoNT/A for both Calu-3 cells (HC 53% lower than BoNT/A for A→B; 45% lower for B→A) and rat alveolar cells (HC 62% lower for A→B; 17% lower for B→A). The transcytosis process was shown to involve an active-energy-dependent mechanism and was significantly inhibited by toxin preincubation with immune serum (Park and Simpson, 2003).

An important caveat to consider when evaluating the relevance of these *in vitro* studies is the use of pulmonary adenocarcinoma and alveolar epithelial cell lines in modeling respiratory absorption. It is generally believed that systemic absorption of inhaled particles is more likely to occur within the distal regions of the respiratory tract; therefore, particles must pass through thinner membranes in the deep lung and are less susceptible to nonabsorptive particle clearance (Palm *et al.*, 1956; Lamanna, 1961; Schlesinger, 1989). Some potential also exists for significant particle absorption from the nasopharyngeal and tracheobronchial regions of the respiratory tract; the cell lines utilized in these *in vitro* studies clearly do not account for this absorption potential. Importantly, investigators in the field have recently sought to characterize the specific cell types involved in toxin absorption from the respiratory tract.

M cells are found not only in the intestinal tract, but also in the respiratory epithelium overlying bronchus-associated lymphoid tissue. The studies of Park and Simpson (2003) indicate that M cells are not the major players in transepithelial transport of toxin across the respiratory epithelium. Additional studies directly investigating the absorption of inhaled BoNTs do not exist.

E. Toxin Binding and Uptake into Target Tissues

The remaining steps of BoNT pathogenesis following neurotoxin absorption are thought to be similar for both the respiratory and gastrointestinal exposure routes. After oral or inhalational exposure, the neurotoxins are absorbed from the gut or respiratory tract, respectively, into the lymphatics and circulation for transport to peripheral cholinergic synapses (Simpson, 2004). Figure 30.3 illustrates the neuromuscular junction, a major target for the actions of BoNTs. BoNTs are taken up presynaptically at the endplate region of neuromuscular junctions (Verderio *et al.*, 1999) and at other cholinergic synapses. Toxin binding involves high-affinity presynaptic receptors. These receptors have recently been identified as a combination of poly-sialogangliosides, synaptic vesicle (SV) protein 2 (SV2), and synaptotagmin (Verderio *et al.*, 2006). Each serotype displays an affinity for a unique combination of receptors. For example, BoNT/B recognizes synaptotagmin II (and I) and ganglioside lipids (Jin *et al.*, 2006; Nishiki *et al.*, 1996; Dong *et al.*, 2003) (see Figure 30.3). BoNT/A involves recognition of SV2C, SV2A, and SV2B (Dong et al., 2003); binding to SV2C also involves a lipid. After toxin binding,

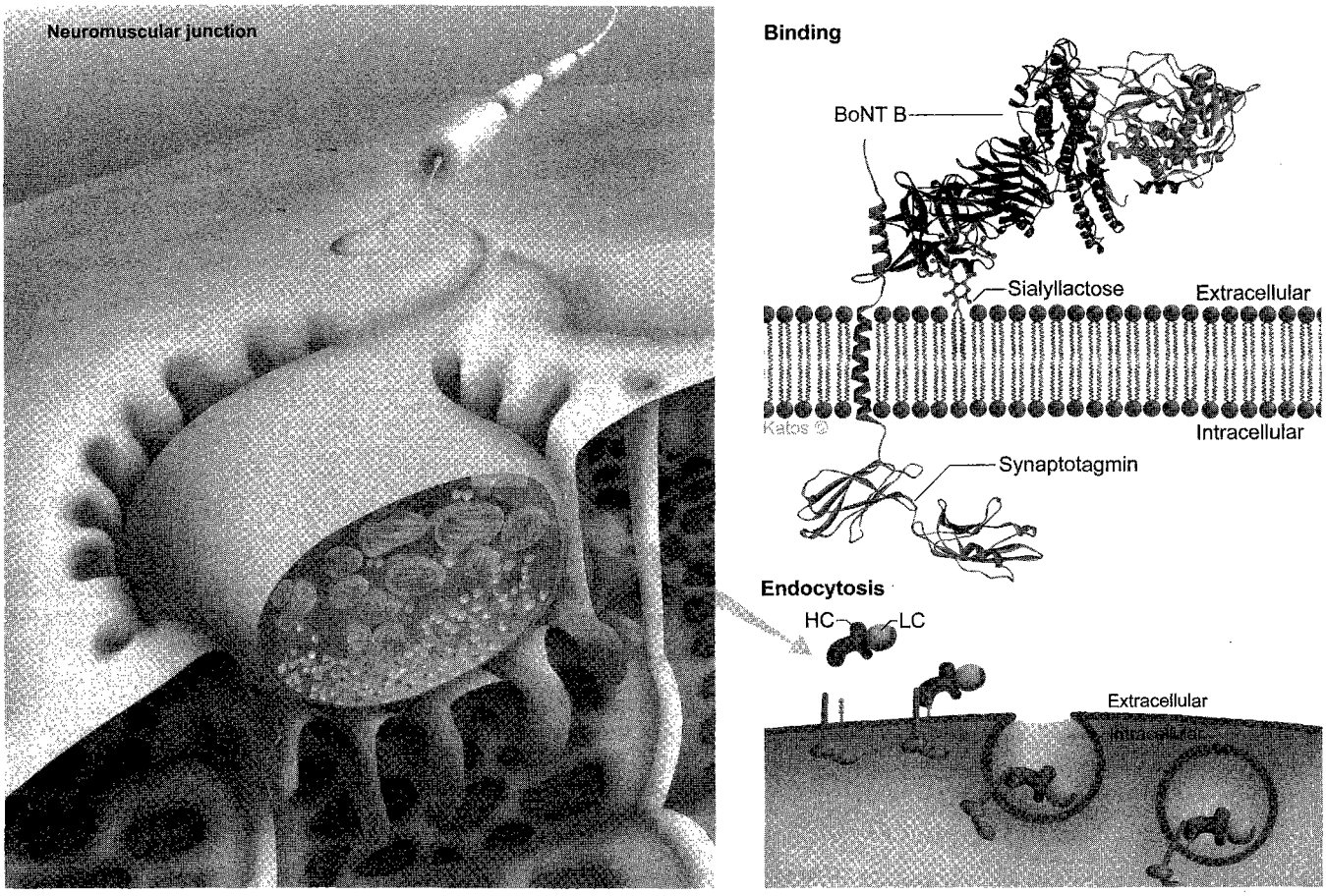

FIGURE 30.3. Toxin binding and internalization at the neuromuscular junction. Left panel: A mammalian neuromuscular junction is illustrated with the alpha motor neuron innervating skeletal muscle at specialized junctional folds in the membrane (Couteaux, 1973). Invaginations of the T system are also illustrated at the level of transition between the A and I bands. The axon loses its myelin sheath and dilates to establish an irregular contact with the muscle fiber. Muscle contraction begins with the release of ACh from synaptic vesicles (tiny spheres) at the motor endplate region. ACh binds to post-synaptic muscle-type nicotinic ACh receptors and causes an increase in the permeability of the sarcolemma. This process is propagated to the rest of the sarcomlemma and ultimately to the sarcoplasmic reticulum (SR) by the T system. An increase in SR permeability liberates calcium ion (Ca^{2+}) stores, resulting in sliding of illustrated muscle filaments and muscle contraction. BoNTs bind and internalize at the presynaptic side of the neuromuscular junction (Verderio *et al.*, 1999). Release of BoNTs into the cytosol results in inhibition of ACh release and flaccid paralysis of innervated muscle. Top right panel (Toxin binding): A three-dimensional ribbon structure of BoNT/B is illustrated. The receptor binding domain of BoNT/B HC binds to synaptotagmin (Syt-II) and ganglioside (sialyllactose) receptors of the presynaptic motor endplate. Each BoNT serotype binds to a different set of receptors in the membrane (Verderio *et al.*, 2006). Bottom right panel (endocytosis): Receptor-mediated endocytosis of BoNT/B holotoxin is illustrated in this panel. The remaining steps in BoNT toxicity involve acidification of the endosome, pH-induced conformational change in the toxin, translocation of BoNT LC across the endosomal membrane with the aid of BoNT HC, and proteolytic degradation of target SNARE proteins by LC. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

the complex is internalized by what is believed to be a clathrin-mediated endocytotic process.

V. TOXICOKINETICS

The onset of symptoms in botulism depends upon the amount of toxin ingested or inhaled and the related kinetics of absorption. Time to onset can range from as early as 2 h to as long as 8 days, although symptoms typically appear between 12 and 72 h after consumption of toxin-contaminated food (Lecour *et al.*, 1988; Arnon *et al.*, 2001). In a review of 13 foodborne botulism outbreaks involving 50 patients from 1970 to 1984, the incubation period ranged from 10 h to 6 days (Lecour *et al.*, 1988).

A. Foodborne Toxicity

1. Toxin Persistence in Circulation and Transit to Target Tissues

Case reports of human foodborne botulism incidents offer some information on toxin persistence and transit after oral exposure in humans. Koenig *et al.* (1964) reported that circulating toxin was detected in five out of six patients suffering from type E botulism after consuming contaminated fish by the mouse lethality assay on serum samples collected from the patients from 1 to 10 days after foodborne exposure. Serum from one of the patients who rapidly succumbed to disease contained approximately 8 $MIPLD_{50}$/ml; extrapolation of this value yields an estimate that 20,000 to 24,000 human LD_{50}s were in this individual's circulation (Koenig *et al.*, 1964). The toxin isolated from the serum of these clinically ill patients was not further activated *in vitro* by trypsin treatment. This observation was in agreement with other studies demonstrating cleavage of the single-chain prototoxin to the active dichain form within the gastrointestinal tract. Importantly, circulating toxin was not detected in a patient with minimal disease 11 days after ingestion of contaminated food (Koenig *et al.*, 1964). Six out of seven individuals who had consumed the contaminated fish but did not develop clinical illness also lacked circulating toxin. Circulating toxin was therefore detected much more consistently in symptomatic patients associated with this outbreak than in subjects who were unaffected after toxin ingestion (Koenig *et al.*, 1964).

Koenig *et al.* (1964) also reviewed previously published literature on the detection of circulating toxin in botulism patients. Circulating toxin (primarily serotype B) had been detected in select patients from 2 to 25 days after consumption of contaminated food, and was rarely detected in type A botulism patients (Koenig *et al.*, 1964). The authors suggested that serotype-specific differences in the persistence of circulating toxin might be attributed to their unique avidities to target tissues. Circulating toxin is generally detected only at very low levels at or immediately prior to death in lethally intoxicated patients (Ono *et al.*, 1970).

Efforts have been made to determine the kinetics of the accessory components of the progenitor toxin complexes after systemic absorption of BoNTs. Iida *et al.* (1970) found that circulating type E toxin was shown to exist in the 7S form after oral administration of the progenitor toxin to rabbits, suggesting that the larger toxin complex dissociated at some point during or after absorption from the gastrointestinal tract. Similar findings were reported on the absorption and persistence of progenitor type A toxin in the rat; the mean sedimentation value of toxin in the lymph after intraduodenal instillation was 7.9S, significantly lower than that of the crystalline toxin (Heckly *et al.*, 1960).

B. Inhalation Toxicity

1. Toxin Persistence in Circulation and Transit to Target Tissues

Very limited data are available on the persistence of BoNTs in circulation following inhalation exposure in any animal species. These data indicate that circulating toxin can be detected soon after exposure but is subsequently cleared rapidly from the circulation. Park and Simpson (2003) reported on the time course for appearance (and amount) of either purified BoNT/A or type A HC in the circulation of mice after intranasal exposure. Maximum serum levels were observed at 2 h post-exposure for both proteins although the peak values were higher for BoNT/A than for HC. Rapid clearance was observed over the next few hours.

An earlier study showed that type A toxin could be detected primarily in the lungs and liver rather than the serum of guinea pigs after intranasal exposure (Sergeyeva, 1962). The same group reported on the correlation between administered toxin dose and detection of toxin in the blood, lungs, and liver of guinea pigs intoxicated via the IN route (Sergeyeva, 1966). Type E toxin was detected in the lungs of guinea pigs after IN administration of two lethal doses, while toxin only appeared in the blood or liver following IN administration of at least five lethal doses. The organ distribution patterns were similar in guinea pigs after inhalation exposure to types A, B, or C toxins (Sergeyeva, 1966). These studies did not address the potential for persistent toxin detection in the lymph after inhalational intoxication, despite the fact that other routes of exposure result in significant absorption into the lymphatics.

While scant literature is available on persistence and distribution after inhalation exposure, several studies have evaluated the systemic behavior of parenterally administered toxins. One group investigated toxin persistence in serum and tissue distribution in white mice following intravenous (IV) administration of 1,000 lethal doses of ^{35}S-labeled type B toxin (Pak and Bulatova, 1962). Mice were sacrificed at 20, 60, and 150 min after toxin administration, and blood and tissues were harvested for toxin distribution analysis. These mice showed symptoms of severe intoxication, including atypical breathing patterns and paralysis, at 150 min post-exposure. Toxin levels (as determined by

radioactivity) were highest in the lung 20 min after toxin injection, followed by the liver, heart, kidneys, intestines, and brain (Pak and Bulatova, 1962). Radioactivity levels in the blood, as well as the liver, heart, intestines, and brain, were further reduced after 60 min post-toxin injection. Serum toxin concentrations were lower than those detected in any other tissue at all time points (Pak and Bulatova, 1962). The authors concluded that the toxin rapidly escaped from blood to various other tissues, suggesting the capacity for unimpeded passage of the toxin through the vasculature and cellular membranes.

Somewhat slower kinetics for toxin clearance from the circulation were observed in dogs following parenteral [IV, IP, or intramuscular (IM)] exposure to type A toxin (House *et al.*, 1964). Serum toxin persistence was evaluated in mongrel dogs receiving 8,000 to 10,000 mouse units/kg of type A toxin. Peak serum toxin levels were detected 5 h after IP administration (13% of injected dose), 12 h after IM administration (9% of injected dose), and within only 3 min after IV administration (79% of injected dose) (House *et al.*, 1964). The relative clearance kinetics were slower after IM and IP exposure than for IV administration, as serum toxin levels were identical 22 h after injection via all three rounds (approximately 6% of injected dose). Some serum toxin activity could be detected by the mouse lethality assay for 2 to 4 days after parenteral administration. Serum toxin patterns were also evaluated in rhesus monkeys following IV administration of type A toxin (Stookey *et al.*, 1965). Serum toxin levels dropped by about 50% of maximum within 16 to 24 h after IV injection, and previous exposure did not affect toxin clearance rates after the administration of subsequent doses.

Another study investigated circulating toxin levels in weanling pigs (5 to 12 weeks old) following parenteral administration of toxin types A, B, C1, and D (Smith *et al.*, 1971). Toxin was cleared from circulation less than 24 h after IV injection of type B (560 $MIPLD_{50}$/kg), type C1 (5,000 $MIPLD_{50}$/kg), or type D (60,000 $MIPLD_{50}$/kg) toxin. In contrast, toxin could consistently be detected in the serum over the entire 4 day period prior to death in pigs injected with serotype A (21,400 $MIPLD_{50}$/kg) (Smith *et al.*, 1971). Serum toxin levels were 100 MIP_{50}/ml at 24 and 48 h after injection of type A toxin, 30 $MIPLD_{50}$/ml after 3 days, and 10 $MIPLD_{50}$/ml after 4 days. These findings indicated serotype-specific differences in the persistence of circulating BoNTs, at least in systemically intoxicated pigs.

Although these studies provide some insight into the persistence of circulating toxin after parenteral administration, they do not necessarily reflect the behavior of absorbed toxin after respiratory exposure. The route of administration may not have a significant impact on the behavior of toxin once absorbed into the serum and lymph, but the patterns and kinetics of absorption into the circulation might be quite different after inhalational versus parenteral exposure. Respiratory exposure could lead to a different proportion of toxin taken up into the circulation and/or lymphatics over a given time period than that seen after systemic injection. Such discrepancies might impact both the quantitative persistence of circulating toxin and its transit to peripheral target tissues. At this point, information is not yet available on the homing and distribution of toxins to target nerve tissues. The available literature also provides no insight on the mechanisms by which toxin is removed from the circulation, either through extravasation and uptake in target tissues or by metabolic processes. In the future, such data will be important in characterizing the pathogenesis of botulism after respiratory intoxication and other routes of exposure.

VI. MECHANISM OF ACTION

ACh release from presynaptic vesicles is dependent upon a propagated action potential, localized depolarization at the presynaptic motor endplate, proper SNARE complex formation (i.e. SNAP-25, syntaxin, and synaptobrevin), and synaptic vesicle docking with the presynaptic membrane (see neuromuscular transmission in absence of BoNT; Figure 30.4). Regardless of the exposure route, BoNTs lead to inhibition in the release of ACh from peripheral cholinergic nerve terminals resulting in flaccid paralysis (Habermann and Dreyer, 1986; Simpson, 1986; see Figure 30.5). The specific target for BoNT/A and/E is the 25 kDa vesicle docking protein SNAP-25; BoNT/A cleaves the last nine residues, whereas BoNT/E cleaves a larger 26 residue fragment from the C-terminus of this protein (Blasi *et al.*, 1993; Montecucco *et al.*, 1994). The target of BoNT/B is the small transmembrane protein synaptobrevin/VAMP located on the surface of small synaptic vesicles (Schiavo *et al.*, 1995). The enzymatically active portion of the 150 kDa BoNT is the 50 kDa light chain; the role of the 100 kDa heavy chain involves binding to cholinergic nerve endings and intracellular penetration via receptor mediated endocytosis (Simpson, 1986, 2004; Monteccuco *et al.*, 1994).

A. Heavy Chain (HC)

The heavy chain of the botulinum neurotoxins has been shown to mediate toxin binding and internalization at cholinergic nerve terminals (Daniels-Holgate and Dolly, 1996; Lalli *et al.*, 1999; Maruta *et al.*, 2004; Simpson, 2004). The mostly β-strand-containing carboxy-terminus of the heavy chain (Hc) appears to be directly involved in toxin binding while the mostly α-helical amino-terminal region (Hn) mediates translocation across the endosomal membrane (Lalli *et al.*, 1999; Simpson, 2004). Through mechanisms that have yet to be fully characterized, the toxins gain entry into the nerve terminal through receptor-mediated endocytosis followed by pH-induced translocation from the endosome to the cytosol. The ability of the Hn region to form transmembrane ion channels raises the possibility that they are intimately involved in translocating the toxic moiety into the cytoplasm (Koriazova and Montal,

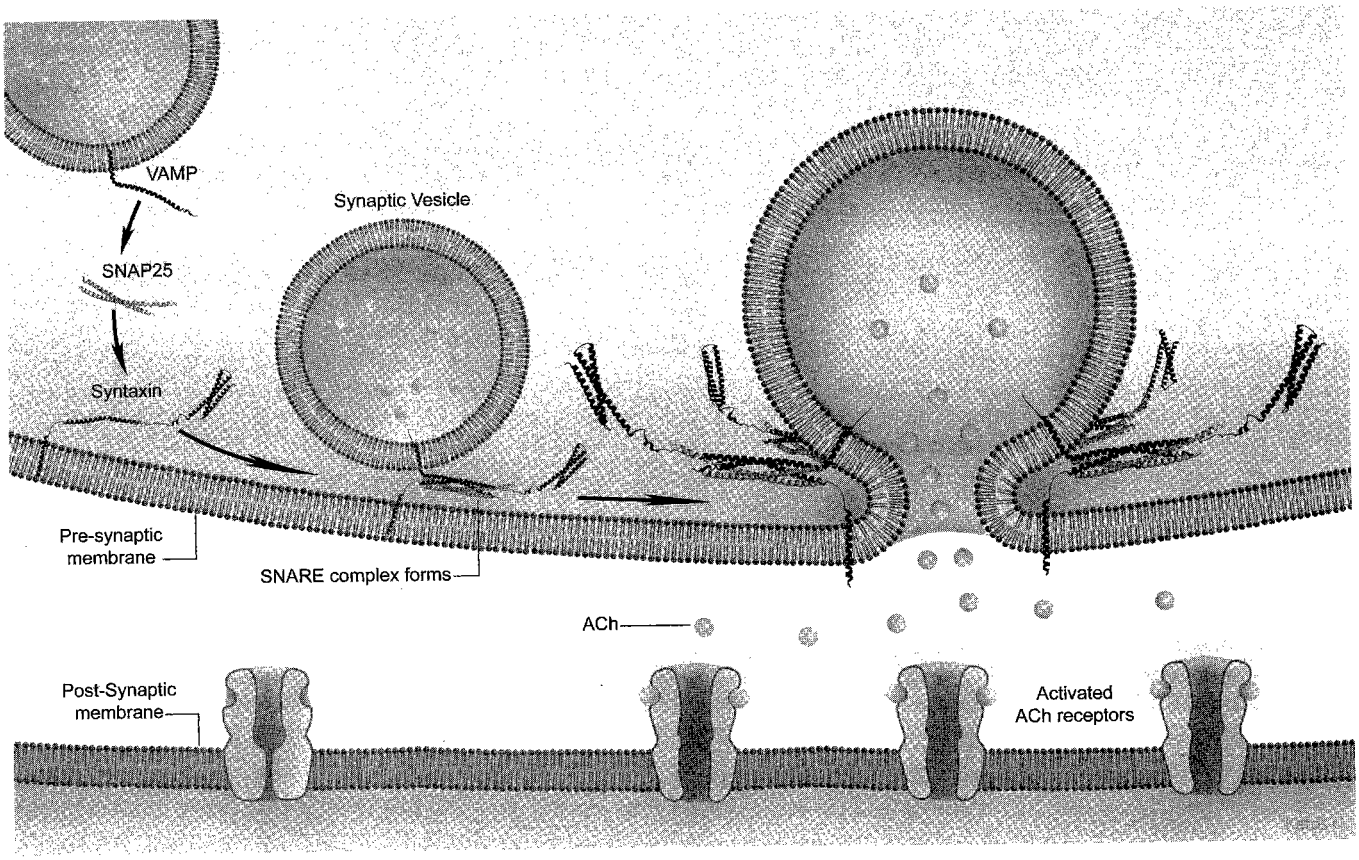

FIGURE 30.4. Neuromuscular transmission in the absence of BoNT. A nerve impulse is transmitted to the effector (muscle) cell by neurotransmitter liberated at the synapse. When the action potential arrives at the axonal terminus to depolarize the presynaptic membrane, Ca^{2+} ions enter through voltage-dependent Ca^{2+} channels. Ca^{2+} ions facilitate the fusion of synaptic vesicles, containing the neurotransmitter ACh, with the presynaptic membrane. Three SNARE proteins (syntaxin, synaptobrevin, and SNAP-25) are critical for synaptic vesicle fusion. As long as the SNARE complex is intact, ACh releases by exocytosis, diffuses across the synaptic cleft, and binds to postsynaptic muscle-type nicotinic ACh receptors. Binding of ACh makes the sarcolemma of the muscle cell more permeable to sodium, which results in membrane depolarization. Excess ACh is hydrolyzed by the enzyme cholinesterase bound to the synaptic cleft basal lamina. ACh breakdown is necessary to avoid prolonged activation of ACh receptors. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

2003). Once translocated into the cytosol, the toxic fragments exert their paralytic effects by inhibiting ACh release from neuromuscular junctions as well as other peripheral cholinergic sites, including sympathetic and parasympathetic ganglia and post-ganglionic parasympathetic synapses (Lamanna, 1959; Vincenzi, 1967; Simpson, 2004).

B. Light Chain (LC)

These paralytic effects have been attributed to the proteolytic activity of BoNT light chain (LC) on protein substrates required for vesicular exocytosis. BoNT LC inhibits neurotransmitter exocytosis through its zinc-dependent endoproteolytic activity. The LCs of the various neurotoxin serotypes possess distinct molecular targets within the peripheral cholinergic nerve termainals (Schiavo *et al.*, 1992, 1993a, b, 1994, 1995; Yamasaki *et al.*, 1994). The endoproteolytic activities of the different toxin LCs produce similar flaccid paralytic effects.

The intracellular proteins SNAP-25, syntaxin, and synaptobrevin (or vesicle-associated membrane protein, VAMP) normally interact with each other in mediating neurotransmitter release from cholinergic and other nerve terminals (see Figure 30.4). Toxin types B, D, F, and G cleave the VAMP proteins while types A, C1, and E act on SNAP-25; type C1 toxin also cleaves syntaxin (Dong *et al.*, 2003). The functions of the various neurotoxins are even more specialized in that one toxin type can cleave its substrate at a different site than that targeted by other toxin serotypes. For example, BoNT/A cleaves SNAP-25 between residues 197 and 198 (resulting in the loss of nine amino acids), while BoNT/E cleaves the same protein between residues 180 and 181 (thereby removing 26 amino acids) (Schiavo *et al.*, 1993a).

Although the LCs of both BoNT/A and/E target SNAP-25, these two serotypes exert significantly different potencies and paralytic profiles in cultured neurons and *in vivo*. A potential molecular basis for this discrepancy was

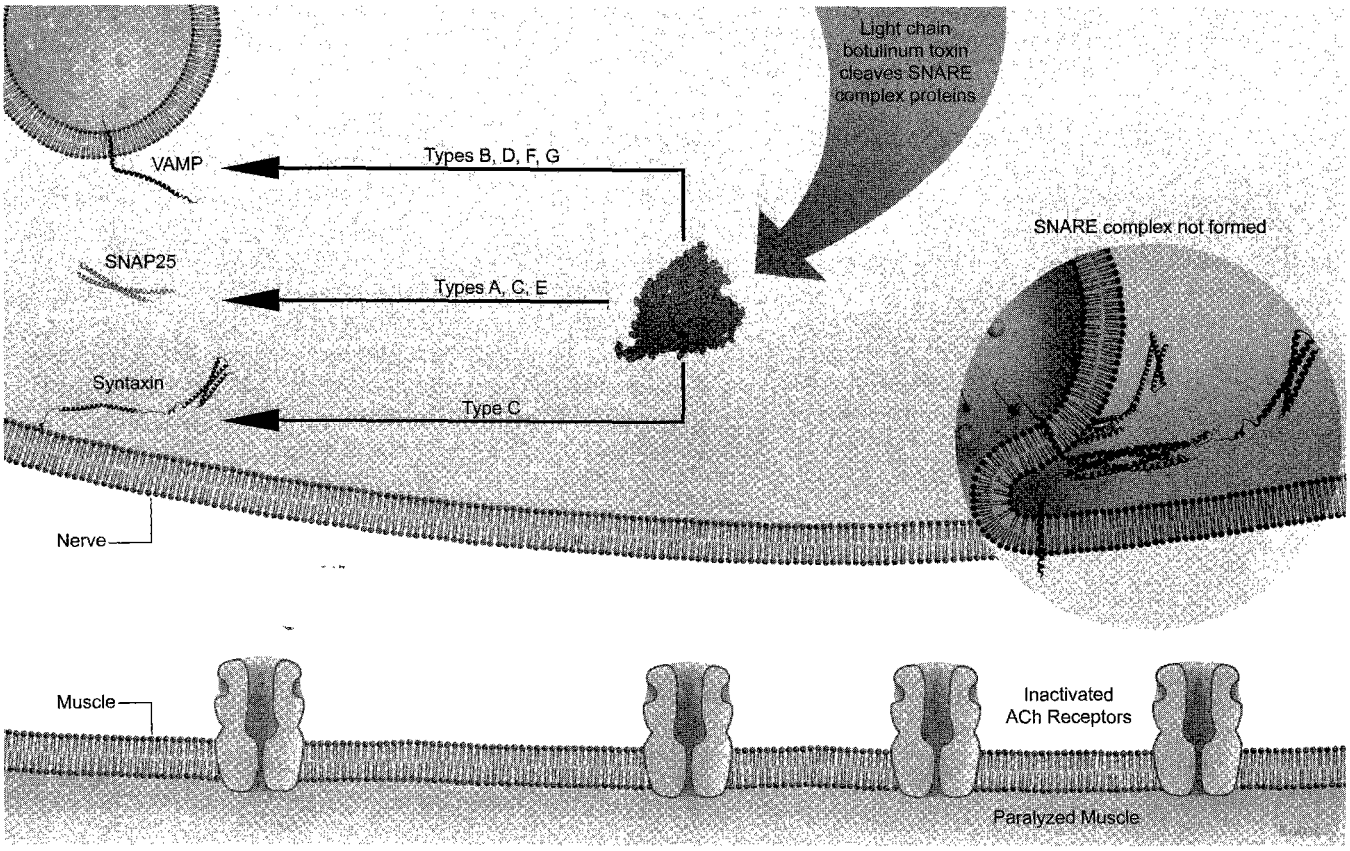

FIGURE 30.5. Neuromuscular transmission in the presence of BoNT. The catalytic LCs of the various serotypes cleave specific SNARE proteins. The SNARE complex does not form if any of the proteins are cleaved. In the presence of BoNT LC inside the axonal terminus, synaptic vesicles will not fuse with the presynaptic membrane, ACh will not be released, and the muscle will not contract, resulting in paralysis at the neuromuscular junction. Illustrations are copyright protected and printed with permission by Alexandre M. Katos.

established by the finding that these neurotoxins target different cleavage sites within the SNAP-25 protein. BoNT/A cleavage generates a 197-residue truncated protein (P197) by cleaving the last nine amino acids from the C-terminus of SNAP-25 while BoNT/E cleavage produces a 180-residue species (P180) by removing the final 26 residues (Schiavo *et al.*, 1993a). A series of studies by Keller *et al.* (1999) and Keller and Neale (2001) provided additional insight into the molecular mechanisms associated with the potent and persistent action of type A neurotoxin relative to type E.

Serotype-specific cleavage events provide insights into the differential activities of the toxins at nerve terminals. In some cases, substrate cleavage studies also revealed important information regarding interspecies differences in the activity of certain toxins. BoNT/B was shown to block neuromuscular transmission by cleaving VAMP proteins between residues Q76 and F77 (in humans and mice) (Bakry *et al.*, 1997). However, the rat VAMP1 (synaptobrevin) protein sequence differs at this critical cleavage site in that the glutamine at position 76 is replaced by a valine, rendering the region more resistant to proteolysis by BoNT/B (Bakry *et al.*, 1997; Verderio *et al.*, 2006; Callaway, 2004). On the other hand, rats and mice were shown to have similar susceptibilities (body weight adjusted) to IM injection of type A toxin; rats have also been shown to be much more resistant than mice to type F toxin (Kauffman *et al.*, 1985).

The specific paralytic profiles associated with each of the BoNTs are typically attributed to their unique proteolytic activities within the nerve terminal. These activities are known to be mediated by the LC components of the various neurotoxins. The various nontoxic components within the multimeric progenitor toxin complexes have traditionally been considered accessory proteins that primarily function to increase neurotoxin stability and, in some cases, to facilitate absorption. Yet studies over the past few years have suggested a potential role for the HA constituents in enhancing the endopeptidase activity of the LC (Cai *et al.*, 1999; Sharma and Singh, 2004). It is widely believed that pure BoNT/A requires either proteolytic "nicking" or chemical reduction for significant SNAP-25 cleavage activity. However, new evidence suggests that the type A progenitor toxin complex is apparently highly active even in nonreduced form (Cai *et al.*, 1999). Further research is needed to substantiate this preliminary work and to establish a more detailed understanding of the prerequisites for LC proteolytic activity.

A recent study by Sharma and Singh (2004) provided additional support for the expanded roles of at least one

neurotoxin-associated protein within the type A progenitor complex. The HA-33 component, representing 25% of the accessory protein content of progenitor neurotoxin, significantly increases the proteolytic activity of both BoNT/A and/E *in vitro* and in rat synaptosome preparations. The addition of HA-33 to nonreduced BoNT/A leads to a 21-fold increase in GST-SNAP25 fusion protein cleavage activity *in vitro* and a 13-fold enhancement of endopeptidase activity in rat synaptosomes (Sharma and Singh, 2004). Similar enhancement of proteolytic activity was seen when HA-33 was added to BoNT/E both *in vitro* and in rat brain synaptosomes.

The enhancement of SNAP-25 cleavage activity by HA-33 in rat brain synaptosomes was taken as evidence that the neurotoxin and the accessory protein both gain entry to the nerve terminal (Sharma and Singh, 2004). The possibility that an accessory component of the progenitor toxin complex could exert direct effects on LC endopeptidase activity within the nerve terminal could have important implications with respect to neurotoxin function *in vivo*.

Two recent reports revealed additional layers of complexity regarding the mechanisms involved in the distinct durations of action associated with the different toxin serotypes. Fernández-Salas *et al.* (2004) investigated the subcellular localization of BoNT/A, /B, and /E LC-GFP fusion proteins following overexpression in several different mammalian cell lines. The LC/A fusion protein was shown to localize within discrete plasma membrane compartments in both neuronal (PC12) and nonneuronal (HEK293, HeLa, HIT-T15) cell lines, while LC/B was detected throughout the cell and LC/E was primarily found within the cytosol.

VII. TOXICITY

A. Lethality

BoNTs are the most potent substances known to man. A comparison of the lethal nature of BoNTs in relation to other toxic chemicals and substances discussed throughout this textbook is provided in Table 30.2. The toxicity associated with oral exposure of a given species to BoNTs is significantly lower than that resulting from parenteral administration (see Table 30.3). The susceptibility of various animal species to parenteral intoxication does not provide adequate indication of their sensitivity to gastrointestinal exposure (Lamanna, 1961). The estimated human LD_{50} of approximately 1 ng/kg for parenteral botulinum intoxication is similar to that reported for most laboratory animals (Arnon, 1995; Middlebrook and Franz, 2000). In contrast, the relative susceptibilities of humans and other animal species to oral intoxication vary significantly (Morton, 1961). A recent clinical review of human botulism reported that the ingestion of as little as 0.05 to 0.1 μg of BoNT A may be sufficient to cause death in humans (Cherington, 1998). Human lethal

TABLE 30.2. Comparison of toxic chemicals and substances

Chemical or toxin	Mouse IV LD_{50}* (mg/kg)	Chemical or toxin	Mouse IV LD_{50}* (mg/kg)
Botulinum toxin	0.00001	Strychnine	0.41
Batrachotoxin	0.002	Potassium cyanide	2.60
Anthrax lethal toxin	0.003–0.005	Mustard[b]	3.30
Ricin	0.005	Aflatoxin	9.5
Tetrodotoxin	0.01	Heroin	21.8
Saxitoxin	0.01	CR[c]	37
VX	0.012	Marijuana[d]	42
Abrin	0.02	BZ[e]	46
GD	0.066	CS[f]	48
GB	0.10	Caffeine[g]	62
GA	0.15	CN	81
TCDD[a]	0.182	Thujone[h] (absinthe)	134.2
Capsaicin	0.40	PAVA[i]	224

*Intravenous dose that is lethal to 50% of mice
[a]2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin (TCDD), a contaminant of the defoliant and herbicide Agent Orange
[b]Mustard gas, 1,1′-thiobis[2-chloroethane]
[c]Riot control agent [dibenz-(b,f)-1,4-oxazepine]
[d]Delta-3,4-trans-tetrahydrocannabinol
[e]Incapacitating agent, 3-quinuclidinyl benzilate
[f]Riot control agent (*o*-chlorobenzylidene malononitrile)
[g]Riot control agent (chloroacetophenone)
[h]Constituent of wormwood absinthe, a popular emerald liquor, 4-methyl-1-(1 methylethyl) bicycle [3.1.0] hexan-3-one
[i]Riot control agent (pelargonic acid vanillylamide)

TABLE 30.3. Comparison of the lethality of serotypes A–G by various routes of administration in the guinea pig[†]

Route of intoxication	Botulinum toxin serotype						
	A	B	C	D	E	F	G
Oral	717	306	177	436	–	–	–
IP[‡]	3.1[a]–5.2[b]	4.2[b]–6.5[a]	1.6[b]–3.2[a]	3.0[c]–6.4[a]	34.3[b]–78[c]	–	40–100[d]
IM[††]	4.3[a]	6.9[a]	3.1[a]	8.7[a]	102[a]	–	–
SC[‡‡]	6[e]–30[f]	–	–	3[g]	100[h]	30–30[g]	–
Aerosol	141	350	87	186	778	–	–

[†]The doses are normalized to mouse IP LD_{50} units
[‡]Intraperitoneal administration
[††]Intramuscular administration
[‡‡]Subcutaneous administration
[a]Gelzleichter *et al.* (1998a)
[b]Cardella *et al.* (1963)
[c]Lamanna (1961)
[d]Cicarelli *et al.* (1977)
[e]Morton (1961)
[f]Sergeyeva (1962)
[g]Dolman and Murakami (1961)
[h]Sergeyeva (1966)

doses have also been extrapolated from primate studies, yielding an oral lethal dose of approximately 70 μg for crystalline type A toxin for a 70 kg human (Arnon *et al.*, 2001). The lethal human respiratory dose is estimated to be 0.7–0.9 μg and the intravenous or intramuscular dose is 0.05–0.15 μg (Middlebrook and Franz, 2000; Arnon *et al.*, 2001).

B. Oral Toxicity

An earlier report suggested that humans are more susceptible than monkeys to type A toxin by the oral route based on toxin dose estimates in foodborne botulism case studies (Morton, 1961). Morton (1961) also summarized the susceptibility of numerous other animal species to oral botulinum intoxication. Mice, monkeys, and guinea pigs were considered highly susceptible while chickens, rabbits, horses, dogs, rats, and cattle were classified as more resistant, and ferrets, minks, and hogs were deemed resistant. The oral lethal doses of type A toxin in guinea pigs (1,000–3,000 MIPMLD) and monkeys (2,000 MIPMLD) (Morton, 1961) are similar to the estimated oral lethal dose for humans (7,000 MIPMLD).

Morton (1961) provided evidence for an estimated human oral lethal dose of much less than 3,500 MIPMLD for type B toxin. This estimate was based upon an earlier report describing a fatal type B human botulism case resulting from the ingestion of 3,500 MIPMLD in toxin-contaminated cheese (Meyer and Eddie, 1951). A man weighing 104 kg consumed approximately 70 g of contaminated cheese; repeated tests of the cheese indicated that it contained only 50 MLD/g of type B toxin. The patient first developed somewhat atypical disease symptoms of nausea, vomiting, diplopia, dysphagia, phagodynia, and instability within 7 h of exposure (Meyer and Eddie, 1951). The man was later hospitalized and developed symptoms more characteristic of foodborne botulism within 18 to 20 h. He died 57 h after toxin ingestion, despite receiving 35,000 units each of both type A and B antitoxins (Meyer and Eddie, 1951). Therefore, it was determined that 3,500 MIPMLD is in great excess of one minimum lethal dose of type B toxin for humans due to the rapid onset of illness and severity of disease (Morton, 1961). The same study reported a woman who died from botulism 42 h after consuming a small piece of toxin-contaminated pear.

C. Inhalation Toxicity

Naturally occurring botulism cases in humans and other animal species are almost exclusively associated with the ingestion of toxin- or spore-contaminated foods. The level of knowledge in the published literature on toxin absorption following inhalational exposure is therefore much more limited than that associated with gastrointestinal intoxication. The potencies of inhaled BoNTs have been investigated in several experimental animal species. In an early literature review, Morton (1961) reported comparatively similar ratios (5.9:1) of oral to respiratory toxicity (the comparative lethal doses for toxin administered via the oral versus the respiratory route) for type A toxin in guinea pigs and mice. Iakovlev (1958) concluded that guinea pigs were more susceptible than mice to type A toxin by inhalation because they succumbed to intoxication after a shorter incubation period (1–2 days versus 3–4 days for mice). Several technical reports established more specific guinea pig inhalation toxicity data for serotypes A through E (Jemski, 1960, 1961b).

D. Clinical Toxicity

The natural occurrence of human foodborne and infant botulism translates into a wealth of information on the clinical signs and symptoms of disease. This information can be compared to the array of physiological and pathological findings in various species of experimental animals after oral administration of BoNTs. The ability for inhaled BoNTs to produce illness has also been documented in

humans and in several experimental species. The primary neurophysiological signs and symptoms associated with respiratory exposure parallel those observed in cases of foodborne botulism; however, infants display a unique clinical picture of botulism. In addition, the various toxin serotypes are usually associated with analogous clinical presentations, with the most severe cases of foodborne botulism being caused by the ingestion of type A toxin.

Exposure to BoNT via oral or inhalational routes results in symptoms indicative of an inactive peripheral cholinergic system due to inhibition of ACh release from the nerve terminal. The time to onset of disease is dependent on the amount of toxin ingested and ranges from several hours to a few days after oral exposure (Lecour *et al.*, 1988; Arnon *et al.*, 2001). Prominent signs and symptoms of intoxication common to all serotypes and various routes of exposure include, in order of descending frequency: dysphagia, xerostomia, diplopia, dysarthria, fatigue, ptosis of the eyelids, constipation, arm weakness, leg weakness, gaze paralysis, blurred vision, diminished gag reflex, nausea, facial palsy, dyspnea, emesis, tongue weakness, sore throat, dizziness, dilated or fixed pupils, abdominal cramps, reduced or failed reflexes, nystagmus or involuntary rapid eye movement, diarrhea, ataxia, and paresthesia (reviewed by Arnon *et al.*, 2001).

1. Foodborne Botulism

Human foodborne botulism presents as an acute, symmetric, flaccid paralysis that generally involves multiple cranial nerve (CN) palsies initially, termed bulbar involvement. Early symptoms involve paralysis of the motor components of the CNs. The motor components are derived from cell bodies located in the brain with axons that exit the cranium to control muscles, glandular tissue, or specialized muscle in the heart and gastrointestinal tract. Paralysis by BoNTs lead to ptosis and dilated pupils (CN III), disconjugate gaze and blurred vision (CN III, IV, VI), facial droop or palsy (CN VII), dysphagia, dysarthria, and absence of gag reflex (CN IX, X), tongue weakness (CN XII), and weakness of neck strap muscles (CN XI). Botulism patients typically develop difficulty in seeing, speaking, or swallowing in the early phases of intoxication. As paralysis extends caudally, toxic signs and symptoms include loss of head control, hypotonia, generalized weakness, and flaccid paralysis or floppy appearance (infants and children). In infants and young children exposed to BoNT, floppy appearance and constipation may be the only presenting signs to warrant a diagnosis of BoNT exposure, since obtaining a reliable history may not be possible in this population.

Loss of the gag reflex and dysphagia may require intubation and mechanical ventilation. Deep tendon reflexes are often lost during later stages of intoxication, and death in untreated patients results from airway obstruction or inadequate tidal volume (Arnon *et al.*, 2001). Respiratory failure is the most serious clinical manifestation of botulism, and the decline in mortality associated with foodborne botulism is primarily due to improvements in ventilatory support (Lecour *et al.*, 1988). Around 60% of botulism patients in the USA require mechanical ventilation at some point during their hospitalization and treatment (Varma *et al.*, 2004). In severe botulism cases, as in the Florida physician case involving research grade type A toxin instead of BOTOX® for facial muscle paralysis, respiratory support may be required for prolonged periods of time and autonomic dysfunction may persist for months to years (Mackle *et al.*, 2001).

Other clinical forms of the disease share many of these signs and symptoms. The presentation and duration of disease are coupled to the relative persistence of the toxin in blocking the release of ACh at peripheral nerve synapses. Although untreated botulism is potentially deadly, the availability of antiserum has dramatically reduced the mortality rates for the common clinical manifestations of the disease. Severe cases of foodborne botulism may still require ventilatory support for over a month, and neurological symptoms can sometimes persist for more than a year (Mackle *et al.*, 2001).

2. Infant Botulism

The characteristic symptoms of infant botulism are poor sucking, constipation, generalized weakness, floppy appearance, and respiratory insufficiency (Cox and Hinkle, 2002). Infant botulism may quickly progress to respiratory failure if not treated. The development of the intestinal flora has been demonstrated to suppress germination and growth of *Clostridium botulinum* spores in mice (Sugiyama and Mills, 1978). Ingestion of honey by infants is the classic scenario cited in infant botulism; honey is therefore not recommended in this susceptible population (Spika *et al.*, 1989; Arnon, 1998).

VIII. RISK ASSESSMENT

BoNTs present a very real threat to the public health and are the most toxic substances known to humankind. In a military or bioterrorist incident, intoxication by BoNT is likely to occur by inhalation of aerosolized toxin or by ingestion of contaminated food or beverages (Franz, 1997; Sobel *et al.*, 2004). Although the municipal water systems are considered to be safe from BoNT attacks, due to chlorination and dilution, it is not known whether current water treatments adequately decontaminate the toxin. Furthermore, bottled mineral water and milk (Sobel *et al.*, 2004; Kalb *et al.*, 2005) are obvious targets for terrorist groups. The vulnerability of the nation's milk supply was highlighted in a recent modeling study, where its complex distribution system would magnify the consequences of poisoning by BoNT (Wein and Liu, 2005; Kalb *et al.*, 2005). BoNTs are a serious threat to our national security due to their potency, remarkable stability, and persistence in the body.

Wein and Liu (2005) modeled a bioterror attack using BoNTs on the nation's milk supply. Modeling of toxin for dispersal in a liquid medium has been previously computed in a terrorist scenario (Dembek, 2005) involving a water fountain and contamination at a recreational center (CDC, 1999). Wein and Liu's assessment estimates the amount of toxin required, critically evaluates entry points into the milk supply industry, and details deficiencies in our current detection capabilities required to thwart such an attack (Wein and Liu, 2005).

The most prevalent BoNTs isolated in human botulism are serotypes A, B, and E. The ability of serotypes C and D, in addition to F, to paralyze human skeletal muscle should also be noted (Hilmas, unpublished). Complicating matters is the fact that all BoNTs remain stable in common beverages and retain significant potency for prolonged periods of time (>90 days) at room temperature and in biological fluids (human whole blood and serum) at physiological temperatures (Hilmas *et al.*, 2006b; Williams *et al.*, 2007). In addition, BoNTs possess a remarkable ability to remain within the nerve terminal for extended periods. Keller *et al.* (1999) showed BoNT protein detectable by western blot for 90 days in rat spinal cord cultures.

Stability of the BoNT protein should be considered in an assessment of the threat posed by intentional release of the toxins. In addition to the remarkable persistence of the toxin in biological fluids and beverages described above, BoNT remains a potent environmental threat. BoNT/A was subjected to desiccation to simulate the residue of an intentional release. Following 28 days of drying, the toxin still possessed remarkable paralytic properties (Williams *et al.*, 2007).

The duration of muscle paralysis following intoxication by BoNT/A exceeds that resulting from exposure to other BoNT serotypes (Keller *et al.*, 1999; Robinson and Nahata, 2003; Fernández-Salas *et al.*, 2004). The remarkable persistence of BoNT/A action has led to its widespread use in the treatment of disorders of muscle tone and movement (Jankovic and Brin, 1997). Although a long duration is desirable in clinical use, the prolonged action of BoNT/A would also make intoxication by this serotype difficult to treat, particularly if used as a bioweapon (Franz, 1997). The duration of intoxication by BoNT/E is relatively brief (several weeks), whereas BoNT/B is of intermediate duration (Keller *et al.*, 1999; Blanes-Mira *et al.*, 2004). The basis for the differences in serotype persistence is currently unknown. In any case, a bioterrorist attack, involving the most lethal substance known to humankind, would overwhelm the limited resources (i.e. mechanical ventilators) available to treat botulism patients.

IX. TREATMENT

There are currently seven known antigenic serotypes of botulinum toxin, designated with the letters A through G, whereby antitoxin to one type does not cross-neutralize any of the others. Only early administration of antitoxin antibody in cases of suspected botulism will minimize the neurologic damage but will not reverse any existing paralysis. Paralysis could persist for weeks to months, and the available treatment consists of supportive care including fluids, total parenteral nutrition (TPN), and mechanical ventilation.

A. Antitoxin

The administration of heterologous antitoxin was one of the first therapeutic approaches developed for botulism patients and remains the most effective when initiated in the early stages of intoxication. The primary limitation of antitoxin treatment was established in some of the earliest published reports on experimental botulism. One of these reports evaluated the pathogenesis of oral intoxication and the efficacy of antitoxin therapy in monkeys (Dack and Wood, 1928). Antitoxin treatment was not effective when administered after symptoms of botulism were already apparent, despite the fact that circulating toxin could still be detected in many of the animals.

Oberst *et al.* (1967) investigated the effectiveness of antitoxin therapy, artificial respiration, and supportive treatment in rhesus monkeys after IV type A toxin injection. These treatments were administered to the animals either alone or in combination after signs of intoxication were observed. Only one in six monkeys survived after receiving antitoxin injections alone as treatment for overt intoxication with 2.5 LD_{50} of type A toxin (Oberst *et al.*, 1967). A combination of antitoxin therapy and supportive treatment initiated soon after the development of toxic signs protected eight of ten animals from death after IV injection of 4 to 5 LD_{50}. Artificial respiration prolonged survival in monkeys with respiratory paralysis but was not effective as a primary treatment after lethal intoxication; no animals receiving only artificial respiration survived intoxication with 5 to 24 LD_{50} (Oberst *et al.*, 1967). Untreated animals developed overt signs of intoxication within 20 to 38 h and died 32 to 135 h after toxin injection.

While antitoxin treatment was generally ineffective in experimental animals displaying significant clinical signs, several case studies of foodborne botulism indicated that antitoxin therapy remained potentially beneficial in humans even after the onset of illness. Iida (1963) reviewed the high efficacy of antitoxin therapy in type E botulism outbreaks associated with contaminated fish consumption in Japan. A mortality rate of only 3.5% was observed among 85 antitoxin-treated patients in nine recent foodborne botulism outbreaks, while a rate of 28.9% was reported among 135 untreated patients in 19 previous outbreaks. Iida noted that all moderately and seriously ill patients in a 1962 foodborne type E botulism outbreak survived after antitoxin treatment. Hatheway *et al.* (1984) reported on the effectiveness of trivalent (ABE) antitoxin therapy during a 1978 outbreak of type A botulism. Four of seven patients with confirmed

disease from type A toxin ingestion were treated with two to four vials of trivalent antitoxin (Hatheway *et al.*, 1984). All four treated patients survived, although one of these individuals continued to suffer from severe paralysis and required ventilatory assistance for several months.

The current Centers for Disease Control and Prevention (CDC) therapy for the public is an FDA-approved, bivalent, botulinum equine antitoxin against serotypes A and B. The trivalent antitoxin against types A, B, and E is no longer available. In cases of exposure to any of the other botulinum toxin serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) equine antitoxin, but the time required for typing a toxin subtype would limit its effectiveness in such cases as an outbreak. A parenteral vaccine against the toxin is currently available, but the need exists for newer nonparenteral vaccines that could be administered orally or via inhalation.

B. Treatment for Infant Botulism

Administration of equine antitoxin is not recommended for preexposure prophylaxis. The heterologous serum of antitoxin therapy can lead to a high frequency of adverse reactions. The equine antitoxin available for use in humans has been reported to cause adverse reactions such as anaphylaxis in over 20% of treated patients (Lewis and Metzger, 1980). This problem has been circumvented in the development of a safer approach to the treatment of infant botulism using plasma isolated from human subjects repeatedly immunized with pentavalent toxoid. Equine antitoxin is not used as a treatment for infant botulism due to the high risk of serious adverse reactions and the possibility of long-term sensitization to horse serum-based therapeutics (Arnon, 1998). An antiserum product termed BabyBIG (botulism immune globulin), derived from human volunteers immunized with pentavalent toxoid, is available for infant botulism patients. Intravenous BabyBIG therapy has proven extremely effective in counteracting the toxic effects of *C. botulinum* colonization in infants and in avoiding the risk of adverse reactions to equine antitoxin. It is also most effective when administered within 24 h of a high-dose aerosol exposure to the toxin (Gelzleichter *et al.*, 1998a, b, 1999).

C. Vaccines

There are as yet no FDA-approved vaccines to prevent botulism. An investigational pentavalent botulinum toxoid (PBT) product, developed at Fort Detrick, is available for persons at risk for botulism (i.e. laboratory workers, warfighters). While determined to be safe and immunogenic, PBT is not useful or recommended for post-exposure prophylaxis. Antitoxin titers do not develop until a month after the third dose in the vaccine schedule. PBT is reserved for employees at high risk for BoNT exposure but not the general population. Several factors limit the usefulness of PBT as a vaccine for inoculating the general population. These include a declining potency and immunogenicity in recent years, the need to take multiple doses to maintain titers, and the limited supply of the vaccine.

X. CONCLUDING REMARKS AND FUTURE DIRECTION

The toxicity of botulinum toxins leading to paralysis is due to their ability to block ACh release from peripheral cholinergic nerve endings (Simpson, 2004). Once ingested or inhaled, the toxin binds to epithelial cells, transports to target tissues via the circulatory system, targets the NMJ, and penetrates cellular and intracellular membranes. BoNTs bind to the lipid bilayer of the neuronal cell surface, gain access by receptor-mediated endocytosis, and cleave polypeptides involved in exocytosis of ACh. As a result, botulism leads to a descending flaccid paralysis, starting usually in the bulbar musculature to involve deficits in sight, speech, and swallowing. Paralysis eventually progresses beyond cranial nerve (CN) palsies to include generalized muscle weakness and loss of critical accessory muscles of respiration. If untreated, death is inevitable from airway obstruction secondary to paralysis of pharyngeal, diaphragm, and accessory respiratory muscles, as well as loss of the protective gag reflex.

The CDC recommended therapy for the public is a trivalent equine antitoxin against types A, B, and E. In cases of exposure to other BoNT serotypes, the US Army can provide an investigational heptavalent (ABCDEFG) antitoxin. However, the antitoxins are in limited supply and would need to be retrieved from stockpiles. Therefore, the development of safe and effective post-exposure therapeutic compounds for BoNT intoxication is of paramount importance to serve the requirements of the military and civilian populations. In conjunction with drug discovery efforts, there is a parallel exigency to develop appropriate animal models to test the usefulness of various strategies for protection against BoNT intoxication.

A. Development of Animal Model Test Systems

1. Inadequacies of Current Animal Model Test Systems

Currently, a large number of animal models (mice, rats, guinea pigs, rabbits, and nonhuman primates) have been used for BoNT research, and it is not clear which species is the most appropriate. This is especially problematic since there are marked species differences in the relative potencies of the different serotypes and in their latency of action (effect of BoNT/B in mice, rabbits, guinea pigs vs rats; Erdal *et al.*, 1995; McLellan *et al.*, 1996; Hilmas *et al.*, 2006a). Mice, in particular, are desirable in BoNT research because they offer the most favorable balance between the scientific needs of the experiment and consistency with the

existing literature. A variety of mouse strains and sexes have been used for other BoNT studies. The mouse LD_{50} is still used to quantitate the purity of BoNT batches and is the basis of the international standard used in serum neutralization assays of BoNT antitoxin. The mouse phrenic nerve–hemidiaphragm assay has been used to measure the effect of BoNTs on skeletal muscle contraction and the doses necessary for inhibition are well characterized. The mouse has further advantages over other rodent species like rats.

Rats are not a valuable test system for BoNTs as they are widely recognized as being insensitive to serotype B (Verderio *et al.*, 2006). On the other hand, skeletal muscles of CD-1 mice, Hartley guinea pigs, and New Zealand white rabbits have similarities to humans in that their muscles are sensitive to serotypes A, B, C, D, and E (Hilmas *et al.*, 2006b). *In vivo* and *in vitro* physiological assessments of BoNT action in rat have also proved to show inconsistent and erroneous results. *In vivo* experiments using the rat extensor digitorum longus (EDL) muscle assay showed sensitivities of rat muscle to the B serotype at low doses (10 MU, corresponding to approximately 1–10 pM) (Adler *et al.*, 1996), despite the wide body of literature on the rat to the contrary. In addition, *ex vivo* rat phrenic nerve–hemidiaphragm preparations are insensitive to BoNT/B at even very high concentrations in the nanomolar range (Williams *et al.*, 2007).

Another physiological model to evaluate therapeutic candidates against BoNT intoxication is the rat toe spread assay. The rat toe spread assay is problematic as a model test system. First, it will not allow for the evaluation of therapeutic candidates against the B serotype since rats are insensitve to BoNT/B. Second, the rat toe spread assay does not involve focal application of BoNT; neighboring muscles are paralyzed due to local diffusion of toxin from the site of intramuscular injection. Toe spread in the rat is mediated predominantly by digiti minimi abductor muscles and to a lesser extent by the EDL, the actual muscle injected in the assay. Intramuscular injection of rat EDLs with BoNT will primarily paralyze the EDL and to a lesser extent the digiti mini muscles, the true abductors of toe spread, by local diffusion. Therefore, EDL muscles injected with BoNTs would tend to show an erroneously early recovery of toe spread as the primary effectors of toe spread recover sooner compared to injected EDL muscles. To date, there is no acceptable *in vivo* model to test the efficacy of inhibitory compounds.

2. Advantages of the Mouse Hemidiaphragm Assay

Current approaches to the inhibition of BoNT activity involve a number of strategies, each with potential advantages and disadvantages. Ultimately, model test systems that can incorporate each of these potential approaches are needed to evaluate the relative merit of potential therapeutic compounds. Since the presynaptic terminal is the primary target for BoNTs, a test system based on toxin action at presynaptic terminals is indicated. Such systems should permit testing of all relevant aspects of toxin (internalization, activity, overcoming inhibition of transmitter release), should be simple and reliable, and should permit rapid evaluation of novel therapeutics or their precursor compounds.

Due to the high sensitivity of mammalian synapses to the actions of BoNTs, due in part to the presence of high-affinity binding sites for toxin on the cell surface and to the intracellular presence of the appropriate enzymatic substrates, the test model systems should be of mammalian origin. Muscle is the ideal test system for BoNT since it is the most sensitive *in vivo* target for neurotoxin action. In addition, inhibition of the diaphragm muscle is the proximal cause of death in botulism (Habermann and Dreyer, 1986; Simpson, 1986). Furthermore, a positive result with BoNT on muscle implies that the toxin is correctly folded and the binding, catalytic, and translocation domains are all intact. Enzyme linked immunosorbent assays (ELISAs), on the other hand, detect only components of the toxin and may provide positive results when the toxin has in fact lost its ability to intoxicate (Kalb *et al.*, 2005). The mouse phrenic nerve–hemidiaphragm assay is a favorable model test system to evaluate therapeutics against BoNT-induced paralysis.

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